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Original Article

Disulfiram/Copper Complex Induces Cytotoxicity in Pancreatic Cancer Cells and 5-Fluorouracil-Resistant Cells through Nuclear Factor E2-Related Factor-2 Suppression and Reactive Oxygen Species Modulation

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Background/Aims: Pancreatic ductal adenocarcinoma (PDAC) is a challenging cancer to treat and has a poor prognosis and limited treatment options. In this study, the anticancer effects of disulfiram combined with copper (DSF/Cu) on PDAC cells, including those resistant to 5-fluorouracil, was assessed.

Methods: Human pancreatic cancer cells (BxPC-3 and CFPAC-1) and their 5-fluorouracil-resistant (5FUR) counterparts were treated with DSF/Cu to assess cytotoxicity. Expression levels of nuclear factor E2-related factor-2 (NRF-2) and heme oxygenase-1 (HO-1) were analyzed by reverse transcription quantitative polymerase chain reaction and Western blotting, while intracellular reactive oxygen species (ROS) levels were evaluated using H2DCFDA staining and flow cytometry. The effects of DSF/Cu on protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) signaling pathways were evaluated by Western blot analysis. *In vivo* efficacy was investigated using a xenograft mouse model, in which mice were orally administered DSF (75 mg/kg) and Cu (2 mg/kg) twice weekly for 5 weeks.

Results: We demonstrated that DSF/Cu effectively induced cytotoxicity in both pancreatic cancer cells and their 5FUR counterparts by modulating ROS levels, NRF-2 levels, and associated survival pathways. DSF/Cu treatment significantly decreased NRF-2 expression and reduced ROS levels, specifically in 5FUR cells. DSF/Cu facilitated NRF-2-independent HO-1 expression and differentially modulated Akt and MAPK signaling pathways in pancreatic cancer cells and their 5FUR counterparts. *In vivo* studies using a xenograft mouse model confirmed the antitumor efficacy of DSF/Cu, as evidenced by reduced tumor volumes and NRF-2 expression.

Conclusions: These findings highlight the potential of DSF/Cu as a novel and effective therapeutic strategy for PDAC, specifically for overcoming resistance to standard therapies. **(Gut Liver, Published online July 25, 2025)**

Key Words: Disulfiram; Copper; Fluorouracil; NF-E2-related factor 2; Pancreatic neoplasms

INTRODUCTION

Pancreatic cancer is the sixth leading cause of cancer-related deaths, with a global annual incidence increase of 0.6% to 1%.[1] Pancreatic ductal adenocarcinoma is the most common malignancy, accounting for >90% of cases, with a 5-year survival rate of 12.8%.[2,3] The traditional therapeutic options for pancreatic cancer include surgery, radiotherapy, and chemotherapy. However, only approximately 20% of patients are eligible for surgical resection, with a high recurrence rate. Cytotoxic therapies did not significantly enhance patient survival because of drug resistance.[4-6] FOLFIRINOX, a chemotherapy regimen combining 5-fluorouracil (5-FU), leucovorin, irinotecan, and oxaliplatin, has demonstrated enhanced outcomes but is often associated with high toxicity and drug resistance.[7-9]

Among its components, 5-FU is widely used in treating various cancers, where it induces cytotoxicity by binding to DNA and RNA and activating caspase-6 and phospho-B-cell lymphoma 2. However, resistance to 5-FU remains a significant challenge.[10,11]

Disulfiram (DSF), approved by the Food and Drug Administration in 1951 for treating alcoholism, has demonstrated anticancer efficacy with minimal toxicity in clinical trials. DSF acts as a copper (Cu)-dependent antitumor agent, suppressing the survival of various cancer cell types, including prostate, breast, colon, and melanoma cells.[12-16] The DSF and Cu (DSF/Cu) combination induces apoptosis in cancer cells by enhancing reactive oxygen species (ROS) production and inhibiting nuclear factor-kappa B and nuclear factor E2-related factor-2 (NRF-2) expression.[13,17]

NRF-2, a primary regulator of antioxidant responses, is crucial for cell survival, proliferation, mitochondrial function, proteasomal degradation, and drug metabolism.[18-20] It is activated by pathways, such as extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases, and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt). NRF-2 overexpression and its downstream genes are associated with tumorigenesis and chemoresistance, facilitating cancer cell survival and growth.[19,21-23]

In this study, we explored the anticancer effects of DSF/Cu on pancreatic cancer cells and their 5-FU-resistant (5FUR) counterparts. We assessed whether DSF/Cu modulates ROS levels and the NRF-2 signaling pathway, resulting in apoptosis in both pancreatic cancer cells and their 5FUR counterparts. Additionally, we assessed the potential of DSF/Cu as a promising therapeutic strategy for treating pancreatic cancer cells and their 5FUR counterparts.

MATERIALS AND METHODS

1. Reagents and materials

5-FU (F6627), DSF (86720), and Cu (II) D-gluconate (344419) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against NRF-2 (sc-365949), HO-1 (sc-136960), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-47724) were obtained from Santa Cruz Biotechnology (CA, USA). Antibodies against ERK (#4370), phosphorylated ERK (#4695), p38 (#8690), phosphorylated p38 (#4511), Akt (#4691), and phosphorylated Akt (#4060) were purchased from Cell Signaling Technology (Boston, MA, USA).

2. Cell lines and culture conditions

The human pancreatic cancer cell lines CFPAC-1 and BxPC-3 were purchased from the American Type Culture Collection (Teddington, UK). CFPAC-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza, Walkersville, MD, USA) containing 4 mM L-glutamine, supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco) and maintained at 37°C in a humidified incubator with 5% CO_2. BxPC-3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Cytiva, South Logan, UT, USA) containing 2.05 mM L-glutamine, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and maintained under similar conditions.

To establish 5FUR human pancreatic cancer cell lines, parental BxPC-3 and CFPAC-1 cells were initially seeded at 50% to 60% confluence and treated with 0.1 μg/mL 5-FU for 48 hours. BxPC-3 cells at passage 7 and CFPAC-1 cells at passage 9 were used as the starting parental cells. Subsequently, the cells were passaged every 2 to 3 days while progressively extending the drug exposure time. If the cells survived, the 5-FU concentration was doubled and process was repeated until a final concentration of 2.0 μg/mL was achieved. At each concentration, at least three passages were conducted to ensure the stability of the resistant cell lines. The entire process of establishing resistant cell lines needed approximately 8 to 9 months.[22]

3. Cell viability assay

Cells (1×10^4/well) were seeded into 96-well plates (SPL, Pocheon, South Korea). After 24 hours of incubation, the DSF/Cu was applied for 48 hours. A solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg/mL and incubated for 3 hours at 37°C in 5% CO_2. The formazan crystals were dissolved by adding dimethyl sulfoxide (Duksan, Ansan, South Korea) to each well, and the absorbance was measured at 570 nm using a microplate reader (E-MAX, Molecular Devices, San Jose, CA, USA).

4. Reverse transcription quantitative polymerase chain reaction

Total RNA was isolated using RNAiso Plus (Takara Bio Inc., Shiga, Japan), following the manufacturer's protocol and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan), following the manufacturer's protocol. Subsequently, first-strand cDNA of the target gene was amplified using specific primers with the Takara SYBR Premix Ex Taq II (Takara, Kusatsu, Japan) on an iCycler (Bio-Rad, Hercules, CA, USA).

The primers for quantitative polymerase chain reaction

were as follows: human NRF-2, 5'-GAG AGC CCA GCT TTC ATT GC-3' (forward) and 5'-TTG GCT TCT GGA CTT GGA AC-3' (reverse); human HO-1, 5'-ATG ACA CCA AGG ACC AGA GC-3' (forward) and 5'-GTG TAA GGA CCC ATC GGA GA-3' (reverse); human TYMS, 5'-TCT GGA AGG GTG TTT TGG A-3' (forward) and 5'-TCC CAG ATT TTC ACT CCC TT-3' (reverse); human ABCG2, 5'-CAC TGA GAT TTG GGC TGC TT-3' (forward) and 5'-AGT CAA ACC GGT ACT GCA CTC-3' (reverse); human SOD-2, 5'-AAG TAC CAG GAG GCG TTG G-3' (forward) and 5'-TGA ACT TCA GTG CAG GCT GA-3' (reverse); human NQO-1, 5'-AAA GGA CCC TTC CGG AGT AA-3' (forward) and 5'- CCA TCC TTC CAG GAT TTG AA-3' (reverse); human GAPDH, 5'-AGG GCT GCT TTT AAC TCT GGT-3' (forward) and 5'-CCC CAC TTG ATT TTG GAG GGA-3' (reverse); mouse NRF-2, 5' -CAT GAT GGA CTT GGA GTT GC-3' (forward) and 5'-CCT CCA AAG GAT GTC AAT CAA-3' (reverse); mouse HO-1, 5'-AGG GTC AGG TGT CCA GAG AA-3' (forward) and 5'-CTT CCA GGG CCG TGT AGA TA-3' (reverse); and mouse GAPDH, 5' -AAG AGG GAT GCT GCC CTT AC-3' (forward) and 5'-CCA TTT TGT CTA CGG GAC GA-3' (reverse). Target gene expression was normalized to that of GAPDH, and the relative fold-change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

5. Western blotting

Cells and tissues were lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protease and phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA). Protein concentration was determined using a bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore, Burlington, MA, USA), blocked with 5% skim milk in tris-buffered saline with Tween 20 (TBST) for 1 hour, and incubated with the primary antibody overnight at 4°C. Subsequently, the membrane was washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour. After washing for three times with TBST, the HRP signal was visualized using the ECL Plus chemiluminescence detection kit (Thermo Fisher Scientific), and signals were detected using a Gel Imager (Bio-Rad).

6. Measurement of ROS levels

Intracellular ROS levels were measured using H2D-CFDA dye (Invitrogen, Carlsbad, CA, USA). The dye was added to the culture medium at a final concentration of 50 μM and incubated for 15 minutes. Intracellular fluorescence was observed using an inverted fluorescence microscope (ZEISS, Baden-Württemberg, Germany). Flow cytometry was performed using a BD FACSCalibur flow cytometer (BD Biosciences, Durham, NC, USA).

7. Ethics statement

This study strictly adhered to the Guide for the Care and Use of Laboratory Animals, established by the National Institutes of Health. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the Gachon University College of Medicine, Seoul, Republic of Korea (Approval No. LCDI-2019-0156). All animal experiments were designed to minimize animal use and euthanasia.

8. *In vivo* xenograft mouse model

CFPAC-1 and 5FUR CFPAC-1 cells (CFPAC-1 5FUR) at 1×10^7 cells/100 μL of Matrigel (Corning, NY, USA) were subcutaneously injected into the dorsal region of 5-week-old male BALB/c nude mice. The mice were randomly divided into four groups (n=4). Group 1 mice received olive oil (vehicle control) orally twice a week for 5 weeks post-subcutaneous transplantation with CFPAC-1 cells. Group 2 mice received olive oil orally twice a week for 5 weeks post-subcutaneous transplantation with CFPAC-1 5FUR cells. Group 3 mice were orally administered with DSF (75 mg/kg) and Cu (2 mg/kg) twice a week for 5 weeks post-CFPAC-1 cell transplantation. The selection of DSF and Cu dosages was based on previous studies demonstrating their efficacy in xenograft models.[24-27] Specifically, 75 mg/kg DSF has been validated in a pancreatic cancer model,[24] while another study used the same dose in a breast cancer model.[25] Cu 2 mg/kg has been consistently used across multiple studies, even when DSF dosing varied from 50 to 250 mg/kg.[26,27] Given this evidence, we adopted the 75 mg/kg DSF + 2 mg/kg Cu combination to ensure both efficacy and safety in our model. Group 4 mice received DSF/Cu orally twice a week for 5 weeks post-CFPAC-1 5FUR cell transplantation. Before cell injection, all mice were pretreated with olive oil or DSF/Cu twice a week. Body weights were measured weekly, and tumor sizes were calculated using the formula: 0.5×length×width×width.[12,13,28,29] Five weeks post-injection, all mice were euthanized and tumors were collected for further analysis.

9. Hematoxylin and eosin staining

After fixation for 24 hours in 4% paraformaldehyde, the tumor xenograft tissues were paraffin-embedded, sectioned at 4 μm thickness, deparaffinized, and hydrated. Hematoxylin and eosin staining was performed following the manufacturer's protocol. The stained sections were observed and

photographed under a microscope (Nikon, Tokyo, Japan).

10. Immunohistochemistry

Tumor tissues from the xenograft mice were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. The embedded tissues were sectioned at 4 μm thickness and heated for 1 hour at 65°C to remove paraffin and hydrate. The sections were incubated in a prewarmed antigen retrieval solution for 10 minutes and subsequently rinsed in phosphate-buffered saline (Cytiva). A 3% H_2O_2 solution was applied for 10 minutes at room temperature, followed by blocking with goat anti-rabbit serum solution in 2% bovine serum albumin (Sigma-Aldrich) for 1 hour. The primary antibody, NRF-2 (ab62352, 1:100; Abcam, Cambridge, UK), was incubated overnight at 4°C. Biotinylated goat anti-rabbit IgG was used as the secondary antibody and incubated for 1 hour at room temperature. Subsequently, the sections were incubated with streptavidin-HRP (Vector Laboratories, Newark, CA, USA) for 30 minutes at room temperature and stained with 3,3'-diaminobenzidine solution (Vector Laboratories). The stained sections were observed and captured under a light microscope at 100× and 400× magnification (Nikon).

11. Statistical analysis

All experimental data were expressed as the mean± standard deviation, and statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was determined using the Student t-test, unpaired Fisher exact test, and Mann-Whitney U test. Statistical significance was set at $p<0.05$.

RESULTS

1. DSF/Cu induces cytotoxicity in both 5FUS and 5FUR human pancreatic cancer cells

To assess the effects of DSF on pancreatic cancer, cytotoxicity assays were performed using 5-FU-susceptible (5FUS; BxPC-3 and CFPAC-1) and 5FUR (BxPC-3 5FUR and CFPAC-1 5FUR) pancreatic cancer cells. DSF treatment alone demonstrated minimal cytotoxicity at concentrations ≤10 μM in all 5FUS and 5FUR pancreatic cancer cells (Fig. 1). DSF/Cu exhibits greater cytotoxicity against various solid tumors than that DSF alone.[12,13,15,17] Similarly, DSF/Cu demonstrated significant cytotoxicity in 5FUS and 5FUR pancreatic cancer cells (Fig. 1). These results indicated that DSF/Cu effectively induces cell death in 5FUS and 5FUR pancreatic cancer cells. To further investigate mechanisms underlying 5-FU resistance, we examined the expression of thymidylate synthase (TYMS) and ATP-binding cassette subfamily G member 2 (ABCG2), which are known to contribute to 5-FU resistance,[30-32] in 5FUR cells (Supplementary Fig. 1). TYMS mRNA expression was significantly elevated in both BxPC-3 5FUR and CFPAC-1 5FUR cells compared to their 5FUS cells. In contrast, ABCG2 mRNA expression showed minimal change in BxPC-3 5FUR cells and was decreased in CFPAC-1 5FUR cells. These findings suggest that 5-FU resistance in these cells is more likely associated with alterations in 5-FU metabolism rather than drug efflux mechanisms.

2. DSF/Cu suppresses NRF-2 and modulates ROS levels in 5FUS and 5FUR pancreatic cancer cells

NRF-2 contributes to cancer progression and chemotherapy resistance in various cancers.[20] We assessed the effect of DSF/Cu treatment on NRF-2 expression by measuring its mRNA and protein levels in treated cells. DSF/Cu significantly inhibited NRF-2 mRNA and protein expression in all 5FUS and 5FUR pancreatic cancer cells (Fig. 2A, C, D, and F). In contrast, the expression of heme oxygenase-1 (HO-1), a downstream target of NRF-2, was either enhanced or remained unaltered in DSF/Cu-treated cells (Fig. 2B, C, E, and F).

Additionally, NRF-2 is involved in regulating ROS production.[33,34] Chemoresistant cancer cells often exhibit high levels of ROS that activate antioxidant mechanisms, such as NRF-2—facilitating chemoresistance through metabolic reprogramming and reduced drug-induced oxidative stress that typically results in cell death in chemosensitive cells.[35] BxPC-3 5FUR and CFPAC-1 5FUR cells exhibited higher ROS activity than that of 5FUS cells (Fig. 3A and D, Supplementary Fig. 2A and D). To investigate whether NRF-2 target genes were also upregulated in 5FUR cells, we examined the basal mRNA expression of NRF-2 downstream targets, superoxide dismutase-2 (SOD-2) and NAD(P)H quinone oxidoreductase-1 (NQO-1). BxPC-3 5FUR cells showed increased expression of both SOD-2 and NQO-1 compared to BxPC-3, and CFPAC-1 5FUR cells also exhibited elevated levels of these genes relative to CFPAC-1 (Supplementary Fig. 3).

DSF/Cu treatment resulted in inconsistent ROS levels in 5FUS cells (Fig. 3B and E, Supplementary Fig. 2B and E). However, significantly reduced ROS levels in 5FUR cells than that in untreated controls (Fig. 3C and F, Supplementary Fig. 2C and F). These findings indicated that DSF/Cu effectively reduces NRF-2 expression and ROS activity, specifically in 5FUR cells.

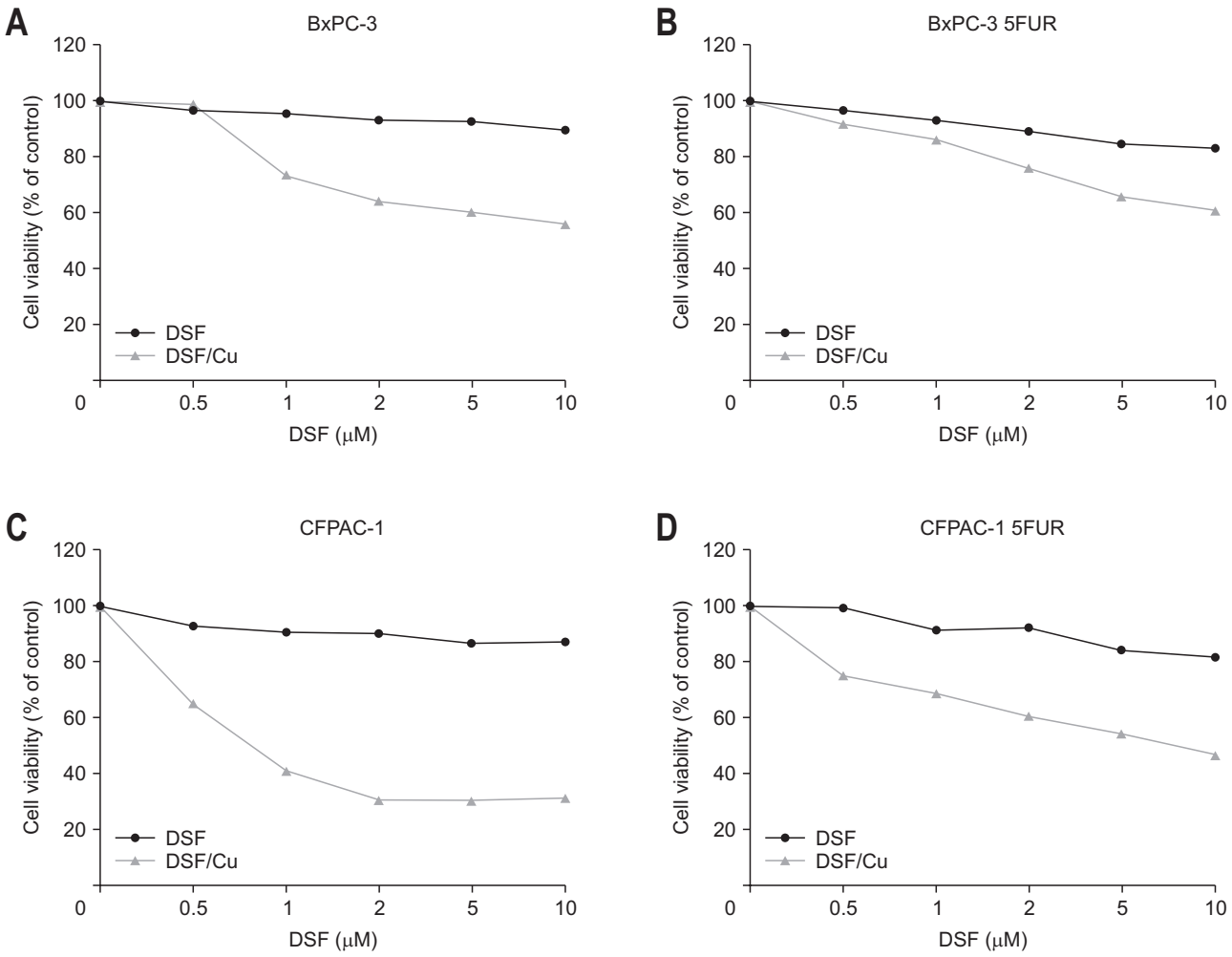

Fig. 1. Disulfiram combined with copper (DSF/Cu) is cytotoxic to 5-fluorouracil-susceptible (5FUS) and 5-fluorouracil-resistant (5FUR) pancreatic cancer cells. (A) Cell viability of BxPC-3 cells treated with DSF alone or in combination with Cu (1 μM) for 48 hours was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B) Cell viability of BxPC-3 5FUR cells treated with DSF, with or without Cu (1 μM), for 48 hours was analyzed using the MTT assay. (C) Cell viability of CFPAC-1 cells treated with DSF alone or in combination with Cu (1 μM) for 48 hours was analyzed using the MTT assay. (D) Cell viability of CFPAC-1 5FUR cells treated with DSF alone or with Cu (1 μM) for 48 hours was analyzed using the MTT assay.

3. DSF/Cu modulates signaling pathways governing cell growth and survival

The Akt and mitogen-activated protein kinase (MAPK) signaling pathways are primary regulators of tumor cell survival and apoptosis.[36-38] To assess the effects of DSF/Cu on these pathways, the phosphorylation of Akt, ERK, and p38 was assessed in DSF/Cu-treated cells. In 5FUS cells, DSF/Cu treatment reduced Akt phosphorylation. Although Akt phosphorylation was unaffected in 5FUR cells, DSF/Cu treatment significantly enhanced ERK and p38 phosphorylation (Fig. 4). Therefore, DSF/Cu differentially modulates signaling pathways in 5FUS and 5FUR pancreatic cancer cells, thereby affecting their survival mechanisms.

4. DSF/Cu enhances antitumor efficacy in an *in vivo* xenograft mouse model

To assess the antitumor efficacy of DSF/Cu *in vivo*, tumor growth was assessed in a xenograft mouse model transplanted with CFPAC-1 and CFPAC-1 5FUR cells. Mice were orally administered equal volumes of olive oil (vehicle) or DSF/Cu twice a week for 6 weeks (Fig. 5A). All mice survived the duration of the experiment, with tumor volumes significantly reduced in the DSF/Cu-treated groups than those in the vehicle-treated groups (Fig. 5B and C). In CFPAC-1 cell-transplanted mice, the DSF/Cu-treated group exhibited significantly smaller tumor volumes compared to that of the vehicle group (603.82±250.06 mm^3 vs 18.68±14.34 mm^3, respectively, $p<0.05$). Additionally, NRF-2 expression in tumor tissues was reduced in the DSF/Cu group compared to that in the vehicle group (Fig. 5D). These findings demonstrated

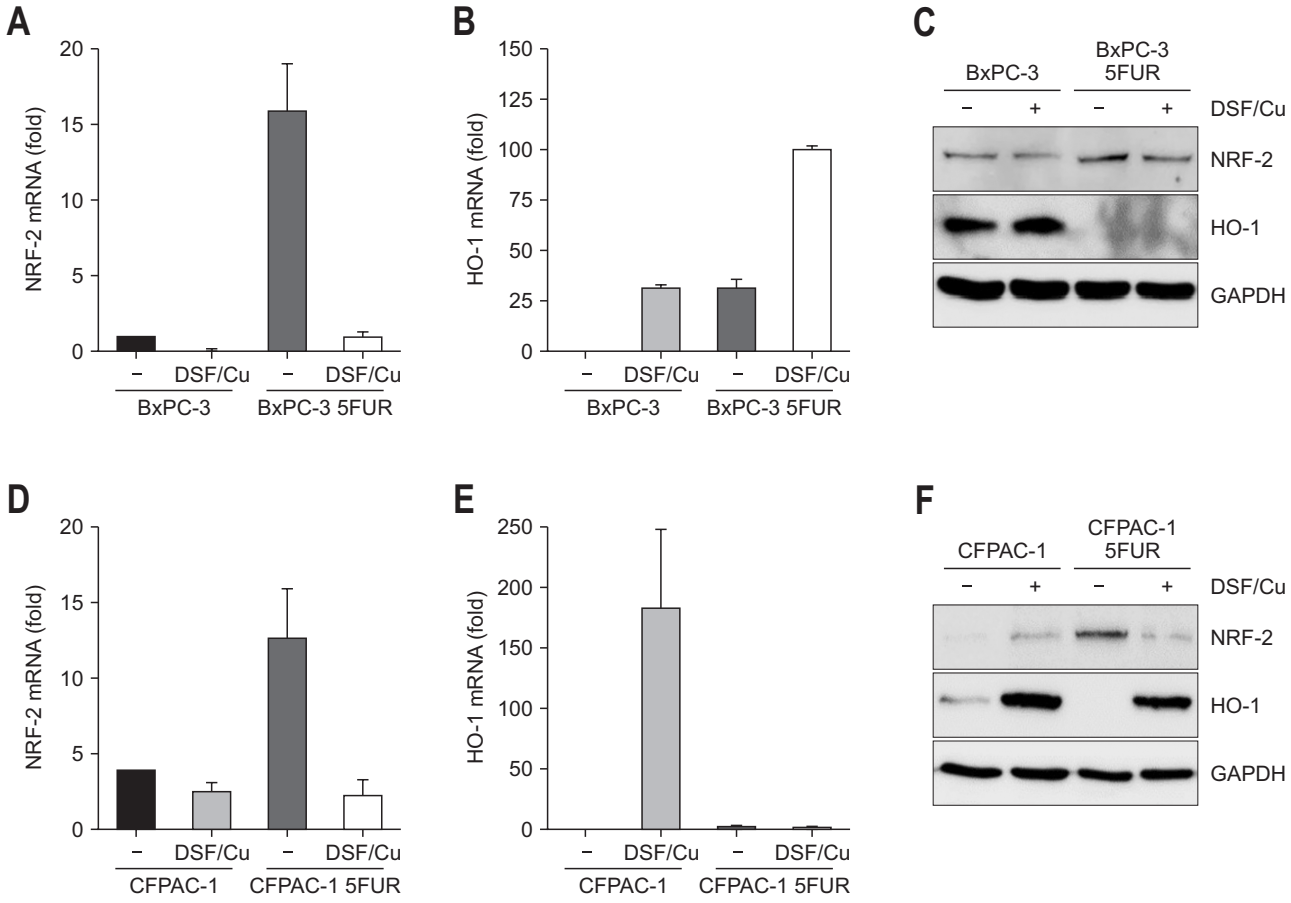

Fig. 2. Disulfiram combined with copper (DSF/Cu) decreases nuclear factor E2-related factor-2 (NRF-2) expression while differentially regulating heme oxygenase-1 (HO-1) levels in 5-fluorouracil-susceptible (5FUS) and 5-fluorouracil-resistant (5FUR) pancreatic cancer cells. (A) Relative mRNA expression levels of NRF-2 in BxPC-3 and BxPC-3 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours were determined using reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B) Relative mRNA expression levels of HO-1 in BxPC-3 and BxPC-3 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours determined using RT-qPCR. (C) Western blotting of whole cell lysates from BxPC-3 and BxPC-3 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours. Protein expression of NRF-2 and HO-1 is analyzed, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as a loading control. (D) Relative mRNA expression levels of NRF-2 in CFPAC-1 and CFPAC-1 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours determined using RT-qPCR. (E) Relative mRNA expression levels of HO-1 in CFPAC-1 and CFPAC-1 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours determined using RT-qPCR. (F) Western blot analysis of whole cell lysates from CFPAC-1 and CFPAC-1 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours. NRF-2 and HO-1 protein levels were analyzed using GAPDH as a control.

that DSF/Cu inhibits tumor growth by suppressing NRF-2 expression in 5FUS and 5FUR pancreatic cancer cells.

5. DSF/Cu regulates Akt and ERK phosphorylation, affecting NRF-2 expression *in vivo*

NRF-2 expression was assessed in tumor tissues from the xenograft mouse model. Similar to the results presented in Fig. 5D, NRF-2 mRNA and protein expressions were reduced in the DSF/Cu-treated groups compared to that in the vehicle-treated groups in both CFPAC-1 and CFPAC-1 5FUR cell-transplanted mice (Fig. 6A and C). While HO-1 mRNA expression was reduced in the DSF/Cu group compared to that in the vehicle group in CFPAC-1 5FUR cell-transplanted mice, HO-1 protein expression was also lower in the DSF/Cu group compared to that in the vehicle group in both CFPAC-1 and CFPAC-1 5FUR cell-transplanted mice (Fig. 6B and D).

Consistent with the *in vitro* findings, in the *in vivo* findings, Akt phosphorylation was reduced in the DSF/Cu-treated groups compared to that in the vehicle groups in CFPAC-1 cell-transplanted mice. In contrast, ERK phosphorylation was enhanced in the DSF/Cu-treated groups compared to that in the vehicle groups in CFPAC-1 5FUR cell-transplanted mice. These results indicated that DSF/Cu treatment may suppress NRF-2 expression by modulating Akt and ERK phosphorylation in 5FUS and 5FUR pancreatic cancer cells.

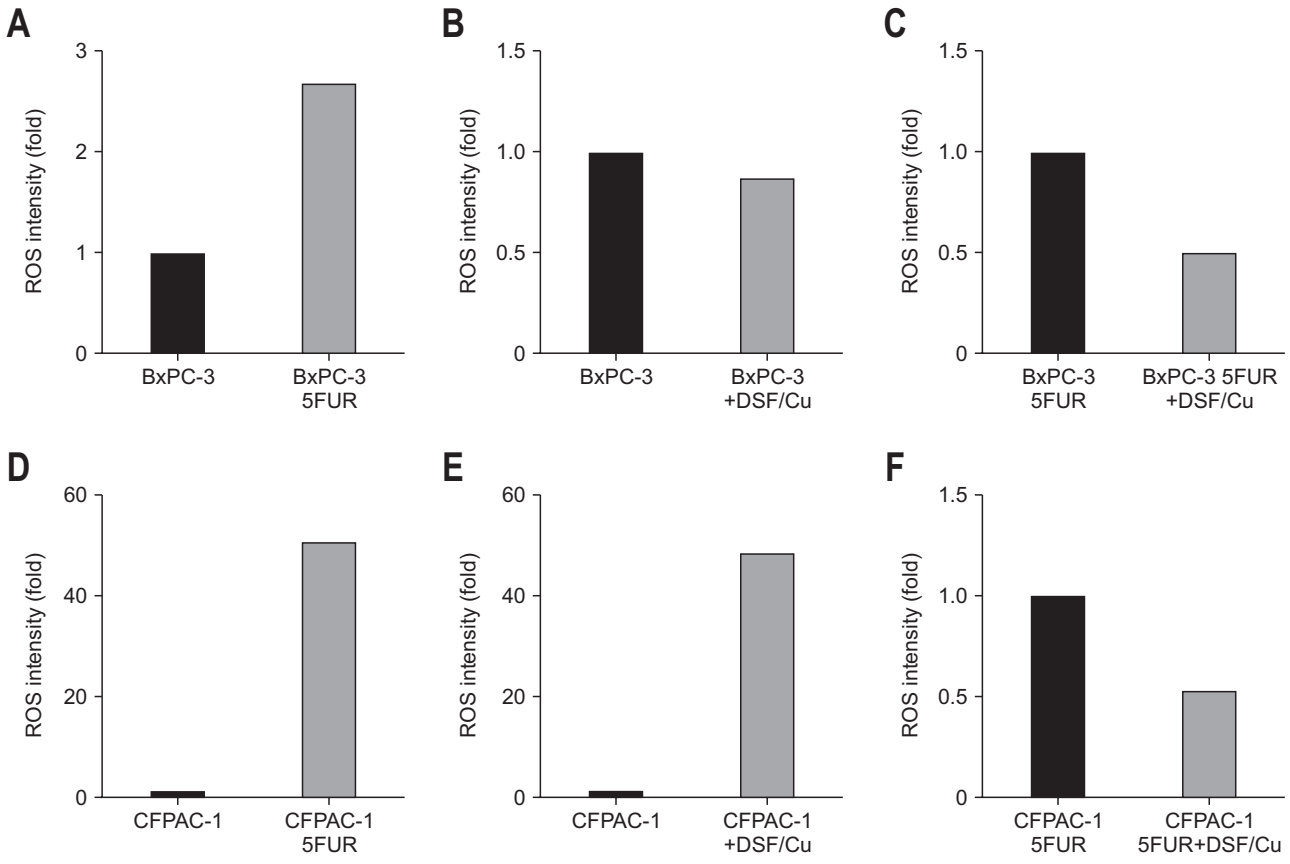

Fig. 3. Disulfiram combined with copper (DSF/Cu) differentially regulates reactive oxygen species (ROS) levels in 5-fluorouracil-susceptible (5FUS) and 5-fluorouracil-resistant (5FUR) pancreatic cancer cells. (A) Comparison of ROS levels between BxPC-3 and BxPC-3 5FUR cells represented as fold-change. (B) ROS levels in BxPC-3 cells after treatment with DSF 10 μM/Cu 1 μM for 48 hours, normalized to untreated controls. (C) ROS levels in BxPC-3 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours expressed as fold-change relative to untreated control. (D) ROS levels in CFPAC-1 and CFPAC-1 5FUR cells expressed as fold-change relative to control. (E) ROS levels in CFPAC-1 cells treated with DSF 10 μM/Cu 1 μM for 48 hours compared to untreated controls. (F) ROS levels in CFPAC-1 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours expressed as fold-change relative to untreated control.

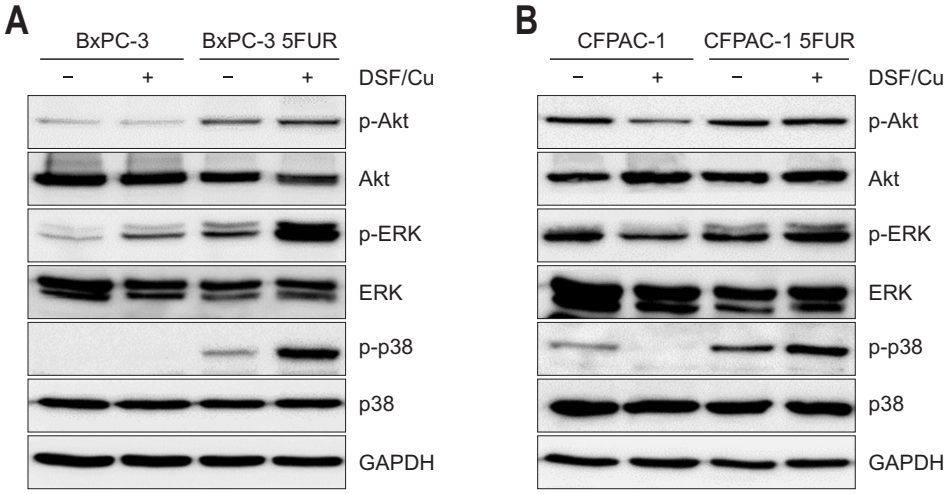

Fig. 4. Disulfiram combined with copper (DSF/Cu) modulates signaling pathways governing cell growth and survival in 5-fluorouracil-susceptible (5FUS) and 5-fluorouracil-resistant (5FUR) pancreatic cancer cells. (A) Analysis of protein expression in BxPC-3 and BxPC-3 5-fluorouracil-resistant (5FUR) cells treated with DSF 10 μM/Cu 1 μM for 48 hours. Western blotting was performed to detect phospho-protein kinase B (p-Akt), Akt, phospho-extracellular signal-regulated kinase (p-ERK), ERK, phospho p38 (p-p38), and p38 levels, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Western blot analysis of CFPAC-1 and CFPAC-1 5FUR cells treated with DSF 10 μM/Cu 1 μM for 48 hours, demonstrating alterations in the expression of p-Akt, Akt, p-ERK, ERK, p-p38, p38, with GAPDH as a loading control.

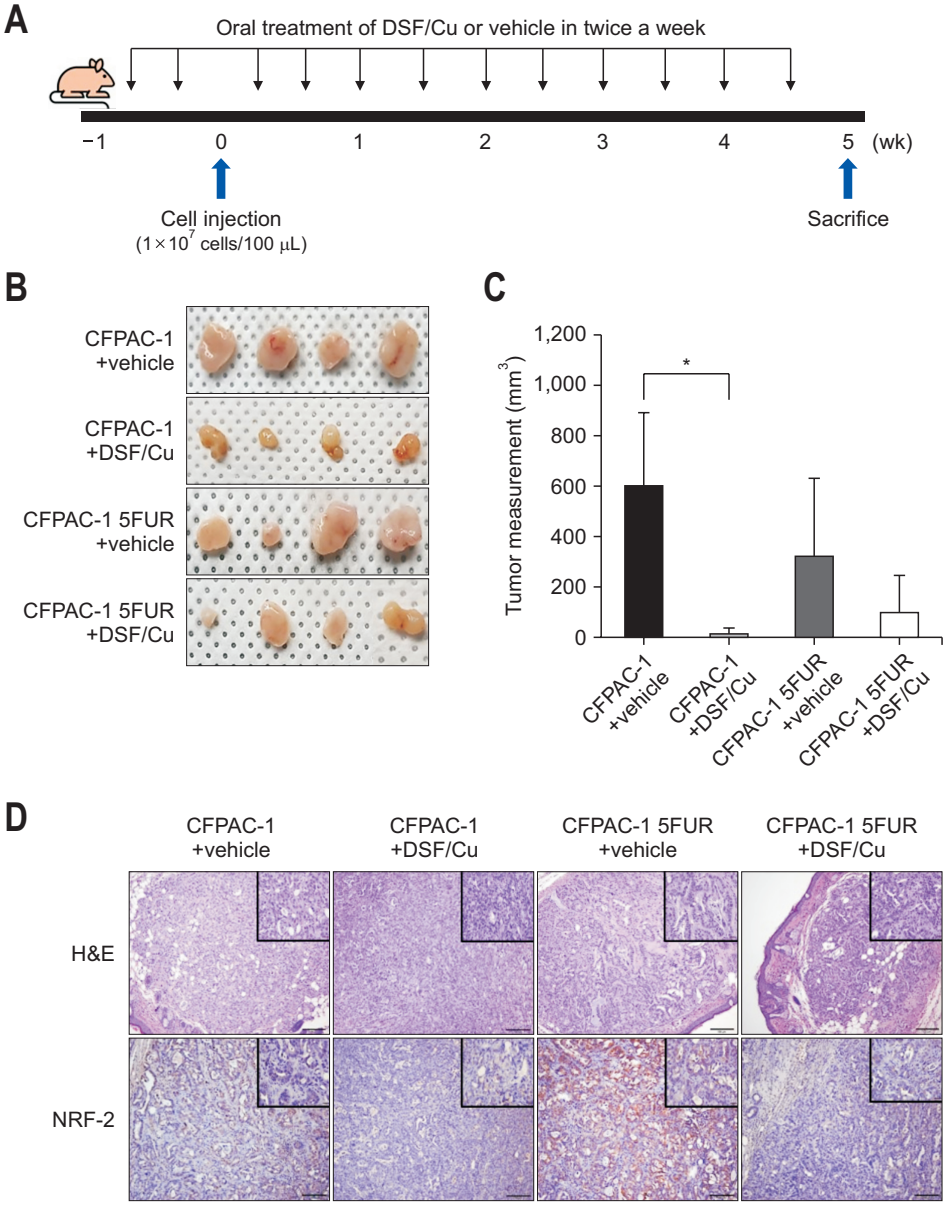

Fig. 5. Disulfiram combined with copper (DSF/Cu) exhibits potent antitumor effects and nuclear factor E2-related factor-2 (NRF-2) downregulation in pancreatic cancer xenograft models. (A) Schematic representation of the experimental timeline for DSF/Cu treatment or vehicle administration in the xenograft mouse model. (B) Representative images of tumors from xenograft mice after 6 weeks of treatment with DSF/Cu or vehicle in CFPAC-1 and CFPAC-1 5-fluorouracil-resistant (5FUR) cells. (C) Tumor volume measurement from xenograft mice after 6 weeks of treatment with DSF/Cu or vehicle (*p<0.05). (D) Immunohistochemical analysis of NRF-2 expression as well as hematoxylin and eosin (H&E) staining in tumor samples of xenograft model mice injected with CFPAC-1 and CFPAC-1 5FUR cells. The scale bar in the large box represents 100 μm (×100), and the inner box represents 20 μm (×400).

DISCUSSION

Although DSF was originally approved by the Food and Drug Administration as an anti-alcoholic drug, recent studies have reported its anticancer effects across multiple cancer types.[39] DSF has demonstrated promising potential in treating cancers, such as prostate,[40] lung,[41] colorectal,[42] and others.[43] However, further studies are required to clarify its role in human pancreatic cancer. DSF/Cu is more effective in various cancers than DSF alone, likely because DSF alone is partially decomposed, limiting its efficacy.[44,45] In this study, we initially assessed the anticancer effects of DSF/Cu in both pancreatic cancer cells and their 5FUR counterparts. Our findings demonstrated that DSF/Cu induces cancer cell death by modulating ROS, NRF-2, and related signaling pathways in both pancreatic cancer cells and their 5FUR counterparts.

In this study, DSF/Cu-induced cell death in both pancreatic cancer cells and their 5FUR counterparts, indicating its potential as a viable therapeutic option for treating both naïve and drug-resistant pancreatic cancers. DSF/Cu overcomes resistance to drugs, such as paclitaxel, gemcitabine, and cisplatin by inhibiting resistance-related targets in cancer cells.[46-48] NRF-2 plays a role in 5-FU chemoresistance in pancreatic ductal adenocarcinoma.[22] NRF-2 inhibition has been associated with enhanced sensitivity to radiation and chemotherapeutic agents, such as doxorubicin, cisplatin, and 5-FU,[49] highlighting the need to explore the interaction between NRF-2 and DSF/Cu treatment in this context. In addition to NRF-2, we also examined other genes impli-

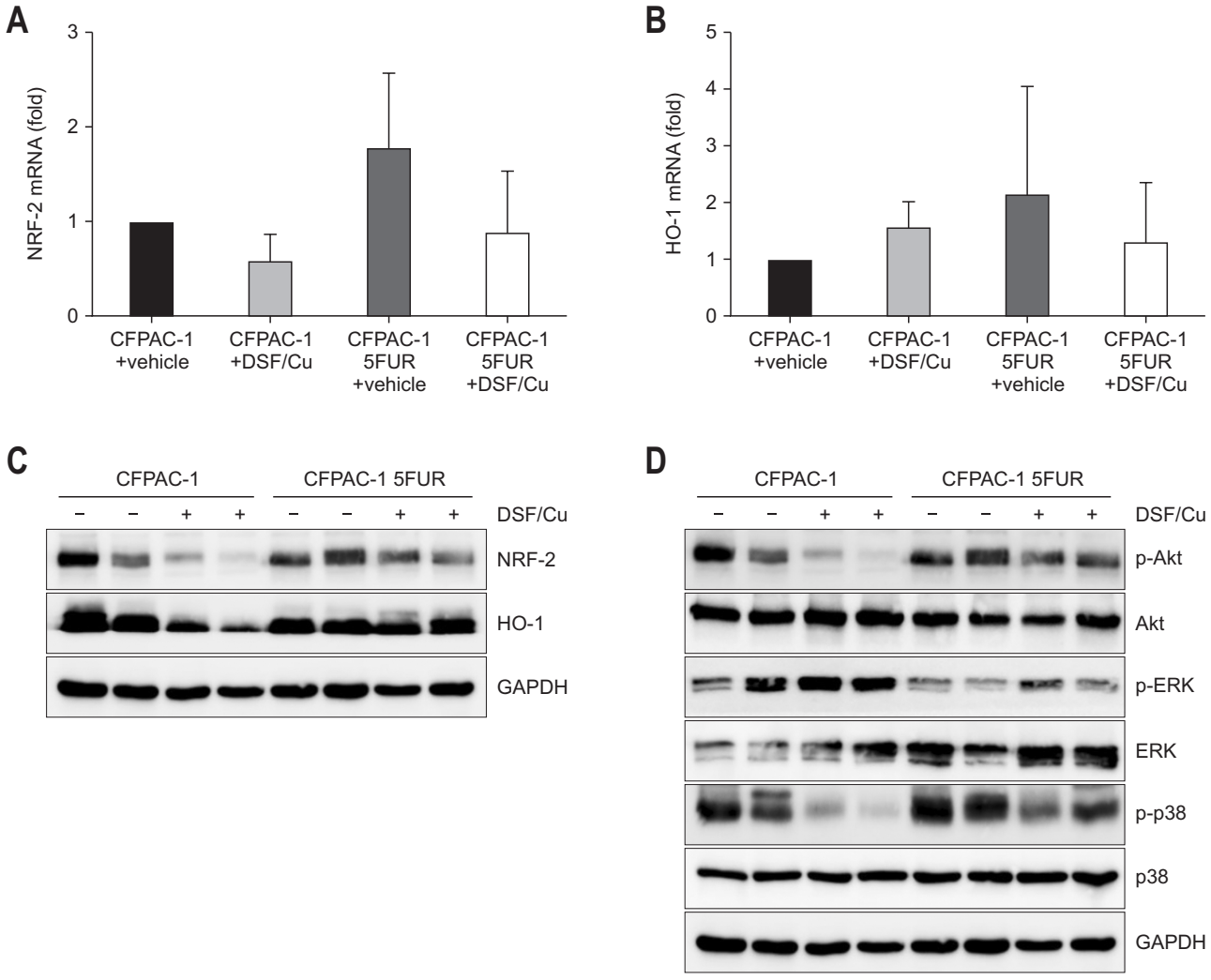

Fig. 6. Disulfiram combined with copper (DSF/Cu) modulates nuclear factor E2-related factor-2 (NRF-2) expression through the regulation of protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) signaling pathways *in vivo*. (A) Relative mRNA expression levels of NRF-2 in tumors derived from xenograft mice treated with DSF/Cu or vehicle, as measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B) Relative mRNA expression levels of heme oxygenase-1 (HO-1) in tumors derived from xenograft mice treated with DSF/Cu or vehicle, as measured using RT-qPCR. (C) Protein levels of NRF-2 and HO-1 are analyzed using Western blotting in tumor tissues of xenograft mice treated with DSF/Cu or vehicle. (D) Western blot analysis of protein expression in tumor tissues from xenograft mice treated with DSF/Cu or the vehicle groups. The expression levels of phospho-protein kinase B (p-Akt), Akt, phospho-extracellular signal-regulated kinase (p-ERK), ERK, phospho p38 (p-p38),, p38, and glyceraldehyde-3-phosphate dehydrogenase (as a loading control) were assessed.

cated in 5-FU resistance. TYMS, the primary target of 5-FU, was found to be upregulated in both BxPC-3 5FUR and CFPAC-1 5FUR cells, which is consistent with its role in conferring resistance to fluoropyrimidines.[50,51] Interestingly, the ATP-binding cassette transporter gene ABCG2, often associated with drug efflux and resistance,[52] showed no significant change between BxPC-3 and BxPC-3 5FUR cells, and was decreased in CFPAC-1 5FUR cells compared to parental controls. These findings suggest that 5-FU resistance in our models may be driven more by enhanced DNA synthesis capacity via TYMS rather than drug efflux mechanisms via ABCG2, and highlight the heterogeneity of resistance mechanisms across different pancreatic cancer cell lines.

ROS production has been implicated in the development of 5-FU resistance because 5-FU increases intracellular ROS levels by inhibiting antioxidant enzymes, thereby inducing chemoresistance in cancer cells. Additionally, NRF-2 is regulated by ROS and is resistant to chemotherapy. In our study, 5FUR cells exhibited significantly elevated basal ROS levels compared to their parental counterparts (approximately >2-fold in BxPC-3 and >40-fold in CFPAC-1), along with increased expression of NRF-2 and its downstream antioxidant genes such as HO-1, SOD-2, and NQO-1. These findings suggest that the resistant cells experience oxidative stress but have adapted via activation of the NRF-2-mediated antioxidant response. Interestingly,

DSF/Cu treatment led to a consistent decrease in ROS levels in 5FUR cells, whereas the response was variable in their parental cells. This discrepancy may stem from differences in basal NRF-2 expression, as BxPC-3 cells exhibit relatively higher NRF-2 protein levels than CFPAC-1, possibly enabling a more robust antioxidant feedback upon DSF/Cu treatment. These results provide mechanistic insights into how DSF/Cu modulates oxidative stress in 5FUR pancreatic cancer cells through differential regulation of the redox response.

In line with this, we observed differential HO-1 mRNA responses to DSF/Cu treatment in BxPC-3 5FUR and CFPAC-1 5FUR cells, with HO-1 being significantly induced only in BxPC-3 5FUR. Although the basal levels of HO-1 mRNA between these two 5FUR cell lines were not directly compared, our experiments indicate that the redox environments and antioxidant gene responses differ substantially between them. This was further supported by flow cytometry analysis, which confirmed substantially higher basal ROS accumulation in CFPAC-1 5FUR compared to BxPC-3 5FUR. Moreover, NRF-2 target genes exhibited distinct expression patterns: SOD-2 mRNA was highly upregulated in BxPC-3 5FUR (>20-fold), while NQO-1 showed greater induction in CFPAC-1 5FUR (>3-fold). These findings suggest that CFPAC-1 5FUR cells may already maintain an elevated antioxidant status, potentially limiting further transcriptional activation of HO-1 in response to DSF/Cu. Conversely, BxPC-3 5FUR cells may retain a more inducible redox response, thereby permitting stronger HO-1 induction upon oxidative modulation.

Our results consistently showed that DSF/Cu treatment led to downregulation of NRF-2 at both mRNA and protein levels across multiple pancreatic cancer cell lines. This reduction in NRF-2 expression may occur through both direct and indirect mechanisms. DSF/Cu may directly suppress NRF-2 transcription or promote degradation of the NRF-2 protein. Alternatively, it may exert indirect effects by modulating upstream regulators such as Kelch-like ECH-associated protein 1 or redox-sensitive signaling pathways. Despite this reduction in NRF-2 expression, HO-1 expression was maintained or even upregulated in both pancreatic cancer cells and their 5FUR counterparts. These findings suggest that DSF/Cu induces HO-1 expression through an NRF-2-independent mechanism. HO-1 expression is known to be regulated by both NRF-2-dependent and -independent pathways. Notably, heme, a substrate of HO-1, has been reported to induce its expression independently of NRF-2.[53] Transcription factors such as activator protein 1 (AP-1), signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and hypoxia-inducible factor 1-alpha (HIF-1α) have also been implicated in NRF-2-independent regulation of HO-1.[54-56] Meanwhile, these pathways were not directly examined in this study, our findings raise the possibility that alternative regulatory mechanisms may contribute to HO-1 expression in this context.

Furthermore, notable discrepancies were observed between *in vitro* and *in vivo* HO-1 expression patterns. While DSF/Cu treatment increased or sustained HO-1 mRNA expression in all tested cell lines, HO-1 protein levels were reduced in CFPAC-1-derived xenograft tumors. These inconsistencies may be attributable to post-transcriptional regulation or the influence of the tumor microenvironment on stress-responsive gene expression. Although the alternative transcriptional pathways were not directly investigated in this study, the observed dissociation between NRF-2 and HO-1 expression supports the hypothesis that DSF/Cu induces HO-1 via NRF-2-independent mechanisms. These findings underscore the need for further studies to elucidate the transcriptional networks governing HO-1 regulation in response to DSF/Cu treatment.

The NRF-2 signaling pathway is modulated by numerous upstream kinases, including MAPK, PI3K, and PKC (protein kinase C).[57] We observed enhanced phosphorylation of ERK and p38 in 5FUR pancreatic cancer cells compared to that in their parental counterparts. Protein kinases, such as PKC, ERK, p38 MAPK, and PI3K participate in the NRF-2 activation pathway, and blocking these kinases has been demonstrated to reduce NRF-2-associated drug resistance.[58] For instance, MAPK inhibition has been reported to enhance the sensitivity of 5FUR colon cancer cells by suppressing proliferation, inducing cell cycle arrest and apoptosis, and reversing multidrug resistance.[59] In this study, DSF/Cu facilitated cancer cell death through distinct signaling mechanisms in pancreatic cancer cells and their 5FUR counterparts. DSF/Cu reduced Akt phosphorylation in parental cells, whereas in 5FUR cells, it enhanced ERK and p38 phosphorylation. The PI3K/Akt pathway typically inhibits pro-apoptotic proteins, reduces apoptosis, and enhances cancer cell survival.[60] Strong and sustained activation of p38 has been associated with apoptosis, senescence, and terminal cell differentiation.[61] Depending on the cell type and stimulus, ERK activation can be associated with intrinsic apoptosis—characterized by cytochrome c release and caspase-9 activation or with extrinsic apoptosis—involving caspase-8 activation.[62] Recent studies have demonstrated that DSF/Cu can induce apoptosis through the ROS/MAPK signaling pathway. However, we observed that DSF/Cu enhanced the phosphorylation of ERK and p38 without affecting the ROS levels in 5FUR cells. This indicated that DSF/Cu may reduce ROS levels in 5FUR cells, potentially enhancing drug sensitivity and inducing ERK

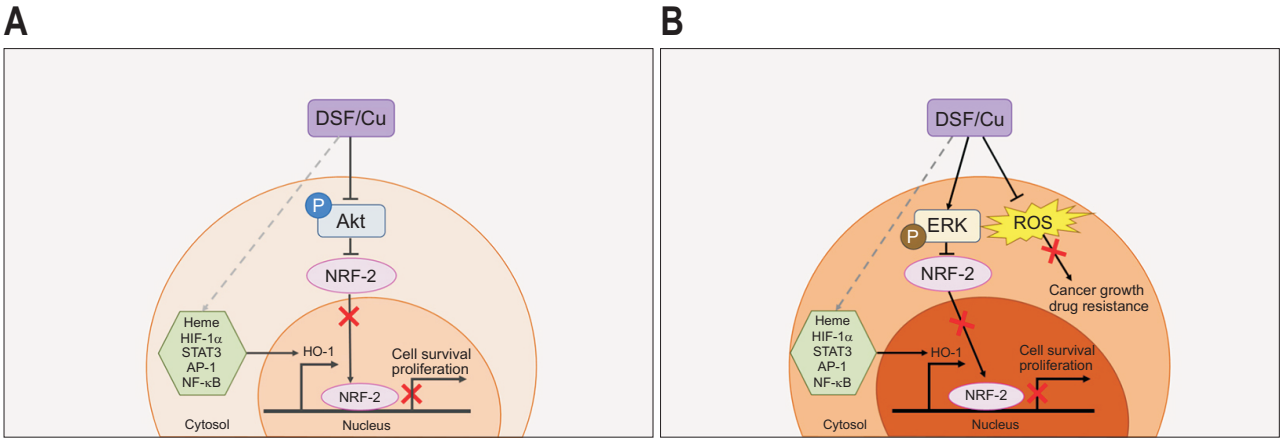

Fig. 7. Schematic representation of the mechanisms through which disulfiram combined with copper (DSF/Cu) mediates signaling pathways in pancreatic cancer cells. DSF/Cu exhibits differential mechanisms in 5-fluorouracil-susceptible (5FUS) and 5-fluorouracil-resistant (5FUR) pancreatic cancer cells. (A) 5FUS cells: DSF/Cu treatment inhibits protein kinase B phosphorylation (p-Akt), resulting in reduced nuclear factor E2-related factor-2 (NRF-2) expression. Although NRF-2 is downregulated, heme or transcription factors such as activator protein 1 (AP-1), signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and hypoxia-inducible factor 1-alpha (HIF-1α) might still induce heme oxygenase-1 (HO-1) expression through an NRF-2-independent pathway. Together, the downregulation of NRF-2 and potential NRF-2-independent HO-1 induction by DSF/Cu may contribute to the induction of cancer cell death. Black solid arrows represent confirmed pathways, while gray dashed arrows illustrate hypothesized mechanisms that have not yet been experimentally validated. (B) 5FUR cells: DSF/Cu treatment promotes extracellular signal-regulated kinase phosphorylation (p-ERK) while reducing the increased reactive oxygen species (ROS) levels that are characteristic of 5FUR cells. Similar to its effects on 5FUS cells, DSF/Cu downregulates NRF-2 expression, whereas heme or transcription factors may maintain HO-1 expression through an NRF-2-independent pathway. The reduction in NRF-2 expression and potential NRF-2-independent HO-1 induction by DSF/Cu may promote cancer cell death, while the reduction in ROS levels may help suppress cancer progression and drug resistance. Black solid arrows represent confirmed pathways, while gray dashed arrows illustrate hypothesized mechanisms that have not yet been experimentally validated. The diagram illustrates the differential effects of DSF/Cu on signaling pathways between 5FUS and 5FUR pancreatic cancer cells, highlighting its potential therapeutic mechanisms in both cell types.

and p38 activation, facilitating cell death.

Consistent with our *in vitro* results, DSF/Cu treatment significantly reduced the tumor volume and NRF-2 expression in an *in vivo* xenograft mouse model. Following DSF/Cu administration, Akt activation was reduced in parental CFPAC-1 cell-injected mice, whereas ERK phosphorylation was enhanced in CFPAC-1 5FUR cell-injected mice compared to that in the controls. Therefore, DSF/Cu may be a potential therapeutic strategy for pancreatic cancer and their 5FUR counterparts (Fig. 7).

This study demonstrates that DSF/Cu suppressed NRF-2 expression and reduced ROS levels, specifically in 5FUR cells, representing a crucial step in overcoming drug resistance. However, certain limitations should be considered when interpreting our findings.

First, this study was conducted using only two pancreatic cancer cell lines (BxPC-3 and CFPAC-1), including their 5FUR counterparts. While these models provide valuable insights, the findings may not be fully generalizable to all pancreatic cancer types. Indeed, we observed some differences between the two cell lines in their response to DSF/Cu, highlighting the potential for cell line-specific effects. Therefore, caution should be exercised in drawing broad conclusions, and further validation using a wider range of pancreatic cancer models is warranted.

Second, our study utilized a xenograft mouse model that is relevant for preclinical drug testing but does not fully recapitulate the complexity of human pancreatic ductal adenocarcinoma, particularly in terms of tumor–immune interactions.[63] Immunocompromised mice lack functional immune components that may influence the therapeutic effects of DSF/Cu; therefore, further validation in more physiologically relevant models, such as patient-derived xenografts or genetically engineered mouse models, is warranted.

Third, although our study provides insight into how DSF/Cu modulates oxidative stress and survival pathways, several mechanistic aspects remain to be clarified. In particular, our findings suggest that HO-1 may be regulated independently of NRF-2. However, we did not directly investigate the involvement of other transcription factors such as AP-1, STAT3, NF-κB, or HIF-1α in this process. In addition, heme, a substrate of HO-1, has been reported to induce HO-1 expression independently of NRF-2 and may represent another regulatory factor contributing to our observations. Further mechanistic studies are needed to explore these potential regulatory pathways. A deeper understanding of these mechanisms may help refine the therapeutic potential of DSF/Cu and identify biomarkers

for treatment responsiveness.

Fourth, the potential off-target effects of DSF/Cu require further investigation. While DSF/Cu exerts its anticancer effects through multiple pathways, including ROS modulation and NRF-2 inhibition, these mechanisms may not be entirely tumor-specific. Further pharmacokinetic and toxicity studies are needed to fully assess its safety profile and potential effects on normal tissues.

Fifth, the optimal dosing regimen for DSF/Cu in cancer treatment may differ from that used in alcohol addiction therapy. Further studies are required to determine the appropriate dosage to achieve antitumor effects while minimizing potential side effects.[24] Moreover, enhancing the drug delivery mechanisms is crucial to enhance the therapeutic efficacy and safety profile of DSF/Cu. Nanoparticle-based delivery systems or other targeted approaches can potentially increase the bioavailability of DSF/Cu in tumor tissues while reducing systemic toxicity.[64] These advancements in drug delivery are essential for overcoming the potential barriers to clinical applications.

Finally, while DSF/Cu demonstrated promise in overcoming 5-FU resistance, the complex nature of chemoresistance in pancreatic cancer may require combination therapies or personalized treatment approaches. Further exploration of the potential synergistic effects with other anticancer agents or targeted therapies yields valuable insights into more comprehensive and effective treatment strategies.[26]

In conclusion, our findings demonstrate that DSF/Cu effectively induces cytotoxicity in both pancreatic cancer cells and their 5FUR counterparts by modulating ROS levels, inhibiting NRF-2, and regulating associated survival pathways. These results highlight the potential of DSF/Cu as a novel therapeutic approach for treating drug-resistant pancreatic cancer and lay the groundwork for future translational research.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: J.H.C. Data acquisition: E.K.K., C.R.J., Y.J.L. Data analysis and interpretation: E.K.K., C.R.J., S.Y.L. Drafting of the manuscript: E.K.K., C.R.J., S.Y.L, Y.J.L. Critical revision of the manuscript for important intellectual content: J.H.C., S.I.J., J.H.J. Statistical analysis: E.K.K., C.R.J., S.Y.L., Y.J.L. Obtained funding: J.H.C. Administrative, technical, or material support: J.H.C. Study supervision: J.H.C. Approval of final manuscript: all authors.

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SUPPLEMENTARY MATERIALS

Supplementary materials can be accessed at https://doi.org/10.5009/gnl250028.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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