Repositioning of aripiprazole, an anti-psychotic drug, to sensitize the chemotherapy of pancreatic cancer

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy with limited therapeutic options. Cisplatin is a primary chemotherapeutic agent utilized in combination with other drugs or radiotherapy for PDAC treatment. However, the severe side effects of cisplatin often necessitate discontinuation of therapy and drug resistance in tumor cells poses significant clinical challenges. Therefore, the development of effective therapeutic strategies is imperative. The present study investigated whether repositioning of the antipsychotic drug aripiprazole could sensitize the anticancer activity of cisplatin in pancreatic cancer at doses calculated by the combination index. The findings indicated that aripiprazole combined with cisplatin to suppress pancreatic cancer cell growth. Notably, the combination notably increased the expression of apoptosis markers, including cleaved caspase-3, compared with cisplatin alone. Additionally, this combination effectively decreased XIAP and MCL-1 expression via mitochondrial membrane potential change as revealed by JC-1 assay, thereby inducing apoptosis. Furthermore, in fluid shear stress assay, the combination of aripiprazole and cisplatin notably inhibited cell adhesion and tumor spheroid formation. Mechanistically, phospho-kinase array profiles showed that the enhanced anticancer efficacy of the combination treatment could be attributed to the inhibition of STAT3 signaling, which led to a significant reduction in tumor growth in a pancreatic cancer animal model. The results showed that the repositioning of aripiprazole inhibits cancer cell growth by blocking the STAT3 signaling pathway and effectively enhancing cisplatin-induced apoptosis, thereby suggesting that the combination of aripiprazole and cisplatin may be a potent chemotherapeutic strategy for the treatment of pancreatic cancer.

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Introduction

Pancreatic cancer is an aggressive malignant tumor and pancreatic ductal adenocarcinoma (PDAC) is the most common form, representing ~90% of all types of pancreatic cancer (1). PDAC is one of the most lethal types of cancer, with a 5-year survival rate of <10%. Of patients with PDAC, ~50% are diagnosed with locally advanced disease, but 85-90% are unsuitable for surgical resection; so the majority receive systemic treatment (2). Despite recent advances in combination chemotherapy regimens based on gemcitabine or 5-FU, the median overall survival for patients with metastatic PDAC is <1 year (3). This underscores the urgent need for novel and effective treatments for PDAC. The standard therapeutic approach for newly diagnosed PDAC involves a combination of surgery and platinum-based chemotherapy.

Cisplatin, a potent platinum-based chemotherapeutics, has been used to treat various solid tumors, including lung, breast, ovarian and PDAC (4). Cisplatin interferes with DNA replication and transcription, leading to cell death, which is particularly effective against rapidly dividing cells such as cancer cells (5). Cisplatin- and platinum-based therapies are commonly employed to treat PDAC, either alone or in combination with other chemotherapeutic agents such as gemcitabine (6). However, despite its clinical efficacy, cisplatin often causes severe side effects, including peripheral neuropathy, hearing loss and kidney damage (7). Therefore, there is an urgent need to develop new pancreatic cancer treatment strategies that do not cause these side effects.

Drug repositioning is a promising strategy for developing new cancer treatments. This approach involves identifying new therapeutic applications for drugs already used in clinical practice, including those approved for other diseases or those unsuccessful for their original indications (8). Repositioned drugs have undergone extensive testing, including safety,

toxicity and pharmacokinetic evaluations, which can expedite their clinical use in cancer therapy at a lower discovery cost (9). More importantly, they can be used in combination with existing cancer therapies to enhance efficacy, overcome drug resistance and improve patient outcomes, thereby providing a route for optimizing and personalizing cancer treatment (10). The synergistic use of repositioned drugs with current treatment regimens offers an avenue to target rare and neglected cancers, providing new hope to patients with limited treatment options.

Markedly, a lower incidence of cancer has been noted in patients with schizophrenia who are prescribed neuroleptics. Some antipsychotic drugs including chlorpromazine and aripiprazole, have demonstrated anticancer effects in preclinical studies (11). Aripiprazole, the second-generation antipsychotic drug for mental disorders, exhibits unique pharmacological activities as a serotonin 5-HT1A and 5-HT2A antagonist. Studies have shown that aripiprazole possesses anticancer properties and enhances radiosensitizing effects in various cancer cells (12-18). It also inhibits the growth of cancer stem cells and overcame the chemoresistance (12). Given the recent emphasis on the importance of sensitization and improving chemotherapeutic resistance in cancer treatment (19), along with the low toxicity of aripiprazole and previous studies suggesting its role in enhancing resistance, it was hypothesized that it could reduce cisplatin toxicity and exert synergistic anticancer effects when combined with cisplatin. To test this hypothesis, the present study investigated the effects of aripiprazole combined with cisplatin on apoptotic cell death and proliferation in pancreatic cancer cells and elucidated its molecular mechanisms involved.

Materials and methods

Ethics statement. Animal protocols were approved by the INHA Institutional Animal Care and Use Committee (INHA IACUC) at the College of Medicine, Inha University (Incheon, Korea; approval nos. INHA 2211124-848 and INHA 230731-883).

Cell culture. MIA PaCa-2 (cat. no. CRL-1420) and Capan-1 cell lines (cat. no. HTB-79) were obtained from the American Type Culture Collection. MIA PaCa-2 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Inc.), while Capan-1 cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco; Thermo Fisher Scientific, Inc.). The medium for MIA PaCa-2 was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. The medium for Capan-1 was supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell culture reagents, including FBS and penicillin/streptomycin, were purchased from Gibco (Thermo Fisher Scientific, Inc.). The cells were maintained in a CO_2 incubator with 95% air and 5% CO_2 at 37˚C.

Chemicals. Aripiprazole and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from MilliporeSigma. For *in vitro* studies, aripiprazole was dissolved in dimethyl sulfoxide (DMSO) at a 20 mM stock concentration and freshly diluted to working concentrations. For *in vivo* experiments, the desired doses were prepared by dissolving in a DMSO:Cremophor:DW (1:2:7) mixture. Cisplatin was purchased from Selleck Chemicals.

Cell viability and proliferation assay. Cell viability was assessed using the MTT assay. Briefly, MIA PaCa-2 and Capan-1 cells were seeded at densities of 2,500 cells and 5,000 cells per well, respectively, in 96-well plates and treated with aripiprazole and/or cisplatin. After 48 h of incubation, 20 μl of MTT solution (2 mg/ml) was added and incubated for an additional 4 h at 37˚C. The plates were read using a microplate reader at a wavelength of 540 nm. The combination index for cisplatin and aripiprazole was calculated using CompuSyn v1.0 (Biosoft), where combination index (CI)<1, CI=1 and CI>1 indicate synergistic, additive and antagonistic effects, respectively (10). Cell proliferation was monitored using the JULI Stage real-time image recording system (NanoEntek).

Colony formation assay. MIA PaCa-2 and Capan-1 cells ($1x10^6$) were treated with cisplatin (1 or 10 mM) and/or aripiprazole (10 mM) for 48 h. The treated cells were seeded in six-well plates at densities of $1x10^3$ and $3x10^3$ cells per well with a monolayer and incubated for 14 days. Colonies were washed twice with Dulbecco's phosphate-buffered saline (DPBS), fixed with 4% paraformaldehyde for 15 min at 4˚C and stained with 1% crystal violet for 15 min at room temperature.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The TUNEL assay was conducted using the ApopTag peroxidase *in situ* apoptosis detection kit (MilliporeSigma). MIA PaCa-2 and Capan-1 cells were treated with cisplatin (1 or 10 μM) and aripiprazole (10 μM) for 48 h, fixed and washed three times with phosphate-buffered saline (PBS). After the terminal deoxynucleotidyl transferase enzyme was activated, the reaction was stopped, and the digoxigenin-conjugated antibody was attached overnight. After washing, 10% 3,3'-diaminobenzidine staining was performed to visualize apoptotic cells and stained for 15 min at room temperature.

Immunofluorescence. The cells were fixed in an acetic acid solution (1:2) for 10 min at 4˚C and permeabilized with 0.5% Triton X-100 for 10 min and incubated in CAS block solution (Life Technologies) for 1 h at room temperature. Next, the cells were incubated overnight at 4˚C with a primary antibody against cleaved caspase-3 (1:50; cat. no. 9661; Cell Signaling Technology). After several washes with PBS, the cells were incubated with fluorescently labeled secondary antibodies (1:60; cat. no. 31460; Invitrogen) for 2 h at room temperature and stained with 4,6-diamidino-2-phenylindole (Antifade mounting medium with DAPI; Vectashield; Vector Laboratories, Inc.) at a 1:100 dilution for 1 h at room temperature. The cells were viewed using a confocal laser scanning microscope (FluoView 1000; Olympus Corporation) at wavelengths of 488 and 568 nm.

Western blotting. The cells were washed three times with ice-cold DPBS before the lysis. Total cellular proteins were extracted using sterile RIPA lysis buffer (Biosesang; 150 mM

sodium chloride, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA), 1X Phosphatase inhibitor (GenDEPOT, LLC; sodium fluoride, sodium orthovanadate, sodium pyrophosphate and sodium glycerophosphate) and 1X Protease inhibitor (GenDEPOT, LLC; PMSF; pepstatin A, leupeptin, benzamidine, bestatin). Equal amounts of proteins (50 μg) were separated by 8% or 12% SDS gel electrophoresis using BCA assay and transferred to PVDF membranes (MilliporeSigma). The membranes were blocked with PBS containing 5% skimmed milk for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies. After washing, the membranes were incubated with secondary antibodies (1:2,000, Mouse (cat. no. 7076S), Rabbit (cat. no. 7074S); Cell signaling Technology, Inc.) for 1 h at room temperature. Proteins were visualized using Clarity Western ECL Substrate (Amersham Biosciences; Cytiva). Primary antibodies from Cell Signaling Technology, Inc. included cleaved caspase-3 (1:1,000; cat. no. 9661), AKT (1:1,000; cat. no. 9272), phosphorylated (p)-AKT (1:2,000; cat. no. 4060), p-SRC (1:1,000; cat. no. 6943), STAT3 (1:1,000; cat. no. 9139), p-STAT3 (1:1,000; cat. no. 94994) and β-actin (1:10,000; cat. no. 4967). The primary antibody for myeloid cell leukemia-1 (MCL-1, 1:500; cat. no. sc-819) and XIAP (1:500; cat. no. sc-55550) was obtained from Santa Cruz Biotechnology, Inc. The densitometry was quantified using ImageJ software (version 1.54, National Institutes of Health).

Measurement of mitochondrial membrane potential by JC-1 Staining. The cells were treated with cisplatin (1 or 10 μM) and/or aripiprazole (10 μM) for 48 h at 37˚C, followed by incubation with JC-1 solution (Cayman Chemical) for 45 min at 37˚C. Following staining with DAPI, the slides were washed twice with DPBS, mounted with Antifade Mounting Medium with DAPI (Vectashield; Vector Laboratories, Inc.) and viewed using confocal laser-scanning microscopy (FluoView 1000; Olympus Corporation).

Two-chamber migration and invasion assay. Cell invasion was analyzed using Transwell permeable support systems (Corning Life Sciences). The inserts were coated with 10% Matrigel for 30 min at 37˚C before cell seeding. Cells were seeded at a density of $2x10^4$ cells/well in a serum-free medium. The lower chambers were treated with cisplatin (1 or 10 μM) and/or aripiprazole (10 μM) for 48 h at 37˚C. After 48 h, the cells that invaded the PET membrane were stained with 0.5% crystal violet for 15 min at room temperature. The migration assay followed a similar protocol, except Matrigel was not used. The purple regions were quantified using ImageJ software (version 1.54; National Institutes of Health).

Fluid shear stress assay. The cells were treated with various concentrations of cisplatin and aripiprazole for 48 h. Cells were then trypsinized and resuspended to a concentration of $1x10^6$ cells/ml in culture medium and subjected to three repeated exposures to shear stress through a 30-gauge needle, followed by a constant flow of 100 μl/s. Briefly, $1x10^6$ cells were resuspended in 4 ml PBS and loaded into a 5 ml plastic syringe. A Luer lock fitting was attached to the end of the syringe and connected to a polyether ether ketone tubing (inner diameter, 125 μm) which was connected to a 50 ml centrifuge tube. The syringe plunger was pushed by a syringe pump to flow the cell solution at flow rates corresponding to wall shear stresses of 5, 20, or 60 dyn/cm^2. The syringe was regularly agitated to maintain cell suspension. After shearing, the cells were centrifuged at 300 x g for 3 min at 4˚C and resuspended in DMEM/10% FBS. The cells were then incubated for 11 days at 37˚C in 96-well ultra-low cluster round-bottom plates (Costar; Corning, Inc.) in a complement medium supplemented with basic fibroblast growth factor, human epidermal growth factor, N-2 and B-27 at 37˚C to form three-dimensional (3D) tumor spheroids. After 11 days, the cells were observed under a phase-contrast microscope and captured images. The size and shape of the 3D tumor spheroids were recorded using an inverted light microscope from day 2 to day 11. Additionally, the cells treated for 48 h were seeded at $8x10^4$ cells per well in collagen-coated 48-well plates, incubated for 1 h at 37˚C, washed twice with DPBS, fixed with 4% paraformaldehyde and incubated for 1 h at room temperature with a 1:100 dilution of DAPI.

Phospho-kinase array. Relative phosphorylation levels of 43 human protein kinases were determined using a human phospho-kinase array kit (R&D Systems, Inc.). MIA PaCa-2 cells were treated with aripiprazole for 12 h at 37˚C and then lysed. After blocking for 1 h at 37˚C with Array Buffer 1 (R&D systems, Inc), the membranes were incubated overnight at 4˚C with 600 μg of protein lysates, washed and then incubated with a streptavidin-HRP detection antibody for 30 min rocking at room temperature. Membranes were developed using ECL Western blotting detection reagents and protein expression levels were quantified using ImageJ software (version 1.54; National Institutes of Health).

Xenograft animal model. Male BALB/c nude mice (five-weeks-old; 18-20 g) were purchased from Orient Bio, Inc. All animal experiments were conducted in accordance with the guidelines of the INHA IACUC (approval no. INHA 2211124-848). The mice were acclimated for one week and then injected with $3x10^6$ MIA PaCa-2 cells into the flank. When the tumor size reached 50-100 mm^3, the mice were randomly assigned to four groups (control, cisplatin, aripiprazole, cisplatin and aripiprazole; n=9). The treatment group received cisplatin [2 mg/kg, intraperitoneally (i.p.)] once a week and/or aripiprazole [10 mg/kg, orally (p.o.)] three times a week. Tumor size was calculated using Vernier calipers with the formula: 0.5x length x (width)^2. After 28 days, the tumors were carefully dissected out and fixed in 10% paraformaldehyde at 4˚C overnight, embedded in paraffin and sectioned at 3 μm. All animals had *ad libitum* access to food and water and were maintained in a stable environment at 25±1˚C, 60±5% humidity and a 12-h light/dark cycle. The health of the mice was monitored by observing the temperature, humidity, noise and lighting conditions in the animal room by monitoring the weight and general condition of mice to assess the health and behavioral status of the mice. At the end of study, the mice were then anesthetized using Ketamin:Rompun (9:1) mixture and sacrificed by collecting blood sample from the heart. The mice were confirmed dead by no spontaneous breathing for 2-3 min and no blink reflex. The blood, lung, liver, pancreas, spleen and tumors of mice were collected for sample preparation.

During the experiment, all mice were anesthetized and sacrificed according to the experimental plan and all measures were taken to decrease the pain of the experimental animals. If the mice showed persistent pain behavior, severe dehydration, inability to eat, extreme fatigue or even severe infection during the research process, they were sacrificed; however, no animals in the present study reached these humane endpoints.

Orthotopic mouse model. Male C57BL/6 mice aged 5 weeks (18-20 g) were purchased from Orient Bio Animal, Inc. All animal experiments were conducted in accordance with the guidelines of the INHA IACUC (approval no. INHA 230731-883). The mice (n=7) were injected with $1x10^4$ KPC cells into the pancreas. After two weeks, the treatment groups received cisplatin (2 mg/kg, i.p.) once a week and/or aripiprazole (10 mg/kg, p.o.) three times a week. After 25 days, the tumors were carefully dissected to avoid contamination from surrounding tissues. At the end of study, the mice were then anesthetized using Ketamin:Rompun (9:1) mixture and sacrificed by collecting blood sample from the heart. The mice were confirmed dead by no spontaneous breathing for 2-3 min and no blink reflex. The blood, lung, liver, pancreas, spleen and tumors of mice were collected for sample preparation. During the experiment, all mice were anesthetized and sacrificed according to the experimental plan and all measures were taken to decrease the pain of the experimental animals. If the mice showed persistent pain behavior, severe dehydration, inability to eat, extreme fatigue or even severe infection during the research process, they were sacrificed; however, no animals in the present study reached these humane endpoints.

Immunohistochemistry. Tumor samples were fixed in 10% buffered formaldehyde at 4˚C overnight, embedded in paraffin, and sectioned, Immunostaining was performed on 3-μm sections of tumor samples after deparaffinization. Antigen retrieval was performed by incubation of 0.2 mg/ml Proteinase K (Thermo Fisher Scientific, Inc.) in PBS for 15 min at room temperature. After gently washing twice with PBS, tissue sections were permeabilized with 0.5% Triton X-100 for 10 min and endogenous peroxidase was blocked with 0.3% H_2O_2 in distilled water for 15 min at room temperature, followed by incubation in CAS block solution (Life Technologies) for 1 h at room temperature. The tissue sections were incubated with primary antibodies (1:50 dilution) following antigen retrieval at 4˚C overnight. Subsequently, sections were incubated with a biotinylated secondary antibody (1:60 dilution; cat. no. 31460) for 1 h at room temperature. Primary antibodies from Cell Signaling Technology, Inc. included cleaved caspase-3 (1:50; cat. no. 9661), BCL-2 (1:50; cat. no. 15071), p-STAT3 (1:50; cat. no. 94994). Primary antibody from Abcam included Ki-67 (1:50; cat. no. ab16667). Immunoreactive proteins were detected by incubating sections with an avidin-biotin peroxidase complex solution ABC kit (Vector Laboratories, Inc.). After washing with PBS, proteins were visualized by incubating sections with DAB for 15 min at room temperature, followed by counterstaining with hematoxylin solution for 40 sec at room temperature. At least three random fields of each section were examined at x400 magnification and analyzed using a computer image analysis system (Olympus Corporation).

Statistical Analysis. All experiments were repeated three times. The data were analyzed using one-way ANOVA with Tukey's post hoc tests for multiple comparisons. Results were presented as mean ± standard deviation (SD). All analyses were performed with GraphPad Prism (version 8.00, GraphPad Software, Inc.; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Aripiprazole enhances the anticancer efficacy of cisplatin in PDAC cells. To identify the synergistic effects of cisplatin and aripiprazole, PDAC cells (MIA PaCa-2 and Capan-1) were treated with both drugs for 48 h. The co-treatment exhibited significant synergistic effects, with CI<1 for the combination of 10 μM cisplatin and 10 μM aripiprazole in MIA PaCa-2 cells (CI=0.884) and 1 μM cisplatin and 10 μM aripiprazole in Capan-1 cells (CI=0.867; Fig. 1A). The cell proliferation curves showed similar trends (Fig. 1B). Additionally, the clonogenic survival of PDAC cells treated with this combination was inhibited in a dose-dependent manner compared with single-agent treatments (Fig. 1C). These findings indicated that aripiprazole enhanced the anticancer efficacy of cisplatin and synergistically inhibits the proliferation of PDAC cells.

Induction of apoptosis in PDAC cells by combination treatment of cisplatin and aripiprazole. Since the co-treatment significantly reduced cell proliferation, it was investigated whether it could induce apoptosis in PDAC cells. TUNEL staining revealed an increase in the percentage of TUNEL-positive cells following treatment (Fig. 2A and B). Furthermore, the combination treatment significantly increased the expression levels of cleaved caspase-3 (Fig. 2C and D). These apoptotic effects were confirmed by the increase in cleaved caspase-3 compared with single-agent treatments, as demonstrated by western blotting (Fig. 2E). Taken together, these results indicated that the combination of cisplatin and aripiprazole synergistically induced apoptosis in pancreatic cancer cells.

Combining of cisplatin and aripiprazole synergistically induces mitochondria-mediated apoptosis. Given that the combined treatment of cisplatin and aripiprazole markedly increased apoptotic cell death, the present study next investigated whether it affects the mitochondrial membrane potential (MMP), which is associated with apoptosis. MMP was examined using JC-1 staining. The control cells exhibited strong red fluorescence in the cytoplasm, whereas the combined treatment showed a clear decrease in red fluorescence and an increase in green fluorescence, indicating changes in MMP associated with apoptosis (Fig. 3A and B). Additionally, the combination treatment decreased the expression of XIAP and MCL-1, prominent anti-apoptotic proteins (Fig. 3C). The results suggested that the synergistic effects of aripiprazole and cisplatin are mediated by mitochondria-mediated apoptosis in PDAC cells.

Combining cisplatin and aripiprazole inhibits cell migration and invasion. Cancer cell migration and invasion are critical steps in metastasis. To investigate the potential inhibitory effects of co-treatment with aripiprazole and cisplatin on these

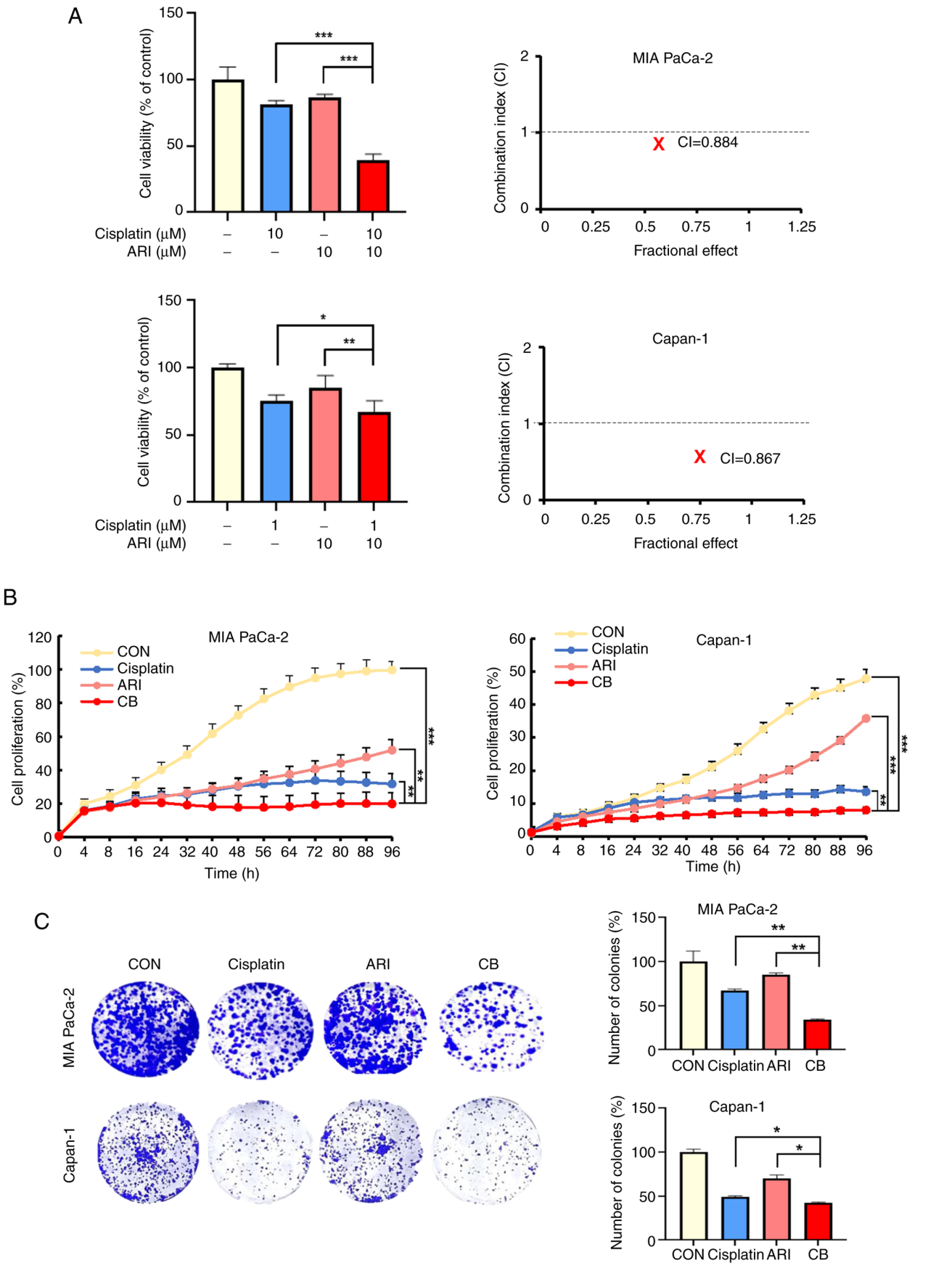

Figure 1. Synergistic cytotoxic effects of cisplatin and aripiprazole in human PDAC cells. (A) MIA PaCa-2 and Capan-1 cells were treated with cisplatin (1 or 10 μM) and/or aripiprazole (10 μM) for 48 h and cytotoxicity was assessed using MTT assays. CI was determined using CompuSyn software (CI<1 indicates synergy). (B) The effect of combination treatment on the cell proliferation was monitored using a JULI stage real-time cell recorder. (C) Colony formation assay was performed in both cell lines. All experiments were performed in three replicates. Data are presented as means ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CI, combination index; CON, control; ARI, aripiprazole; CB, cisplatin and aripiprazole.

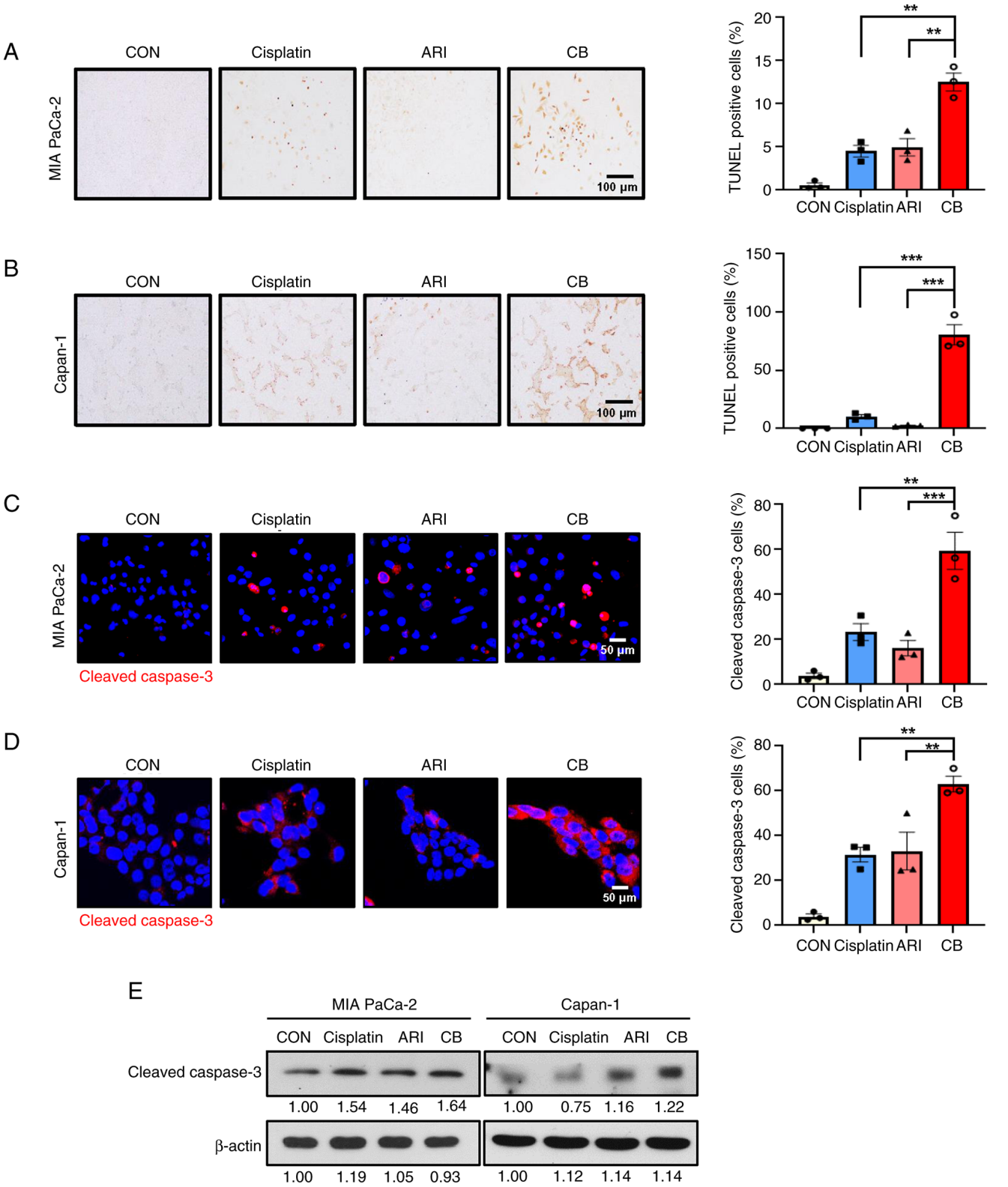

Figure 2. Combined treatment of cisplatin and aripiprazole synergistically induces apoptosis. (A and B) Induction of apoptosis in MIA PaCa-2 and Capan-1 cells by cisplatin and aripiprazole was assessed by TUNEL staining. (C and D) Fluorescence images showing cleaved caspase-3 expression (red) after 48 h of treatment with cisplatin and/or aripiprazole. (E) Western blot analysis of cleaved caspase-3 in cell lysates after 48 h of treatment. All experiments were performed in three replicates. Data are presented as means ± standard deviation. **P<0.01 and ***P<0.001. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; CON, control; ARI, aripiprazole; CB, cisplatin and aripiprazole.

processes, migration and invasion assays were conducted using MIA PaCa-2 and Capan-1 cell lines. Transwell assays demonstrated that the combination of aripiprazole and cisplatin synergistically inhibited both cell migration and invasion compared with the effects of either agent alone (Fig. 4A and B). Additionally, cell spheroid growth and

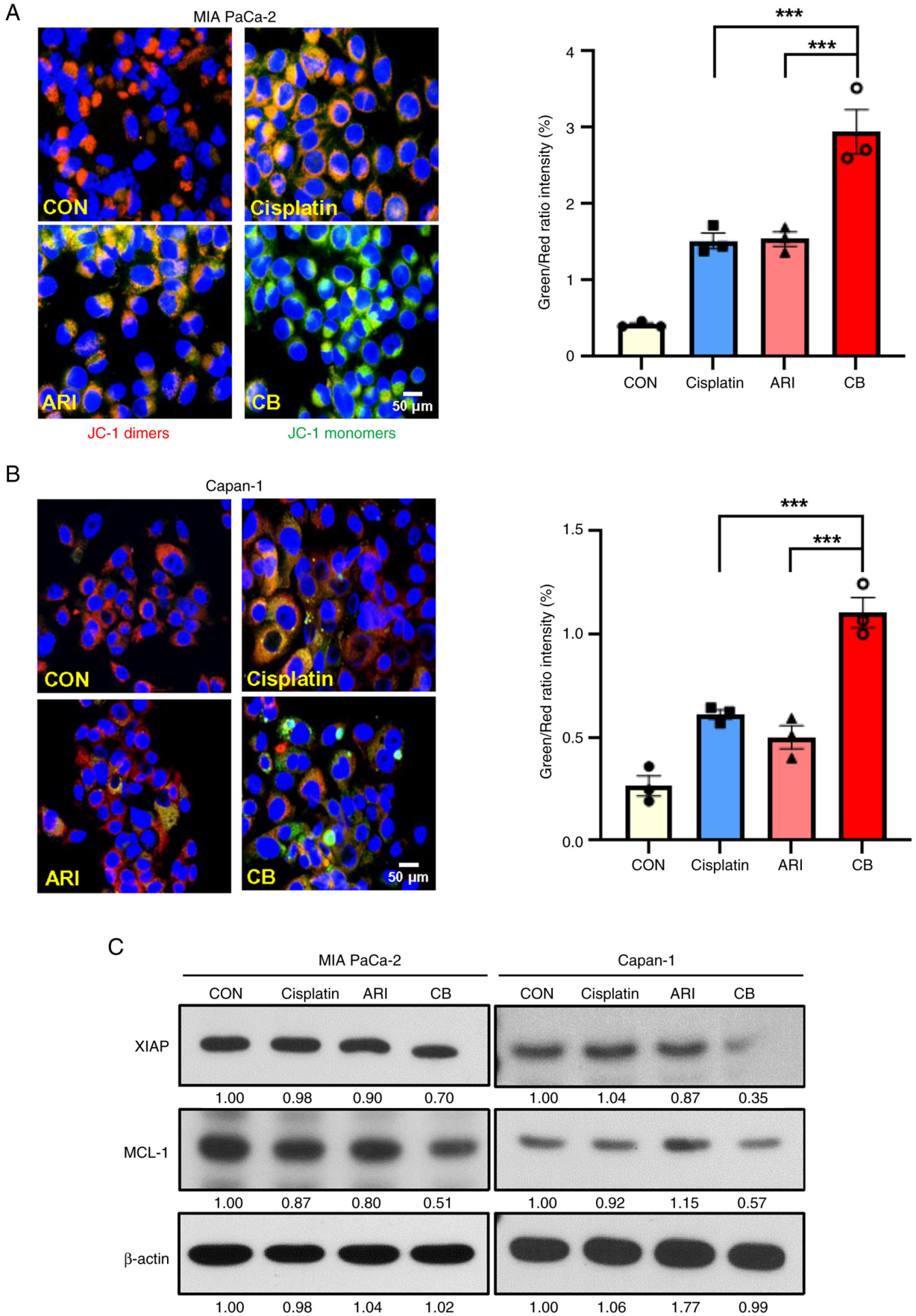

Figure 3. Combined treatment with cisplatin and aripiprazole induces mitochondrial apoptosis. (A and B) Fluorescence images of JC-1 aggregates (red) and monomers (green) in MIA PaCa-2 and Capan-1 cells treated with cisplatin and aripiprazole for 48 h, with the ratio of depolarized to polarized cells plotted. (C) Western blot analysis of MCL-1 and XIAP in cell lysates after 48 h of treatment. Data represents three independent experiments. Data are presented as means ± standard deviation. ***P<0.001. CON, control; ARI, aripiprazole; CB, cisplatin and aripiprazole; MCL-1, myeloid cell leukemia-1; XIAP, X-linked inhibitor of apoptosis protein.

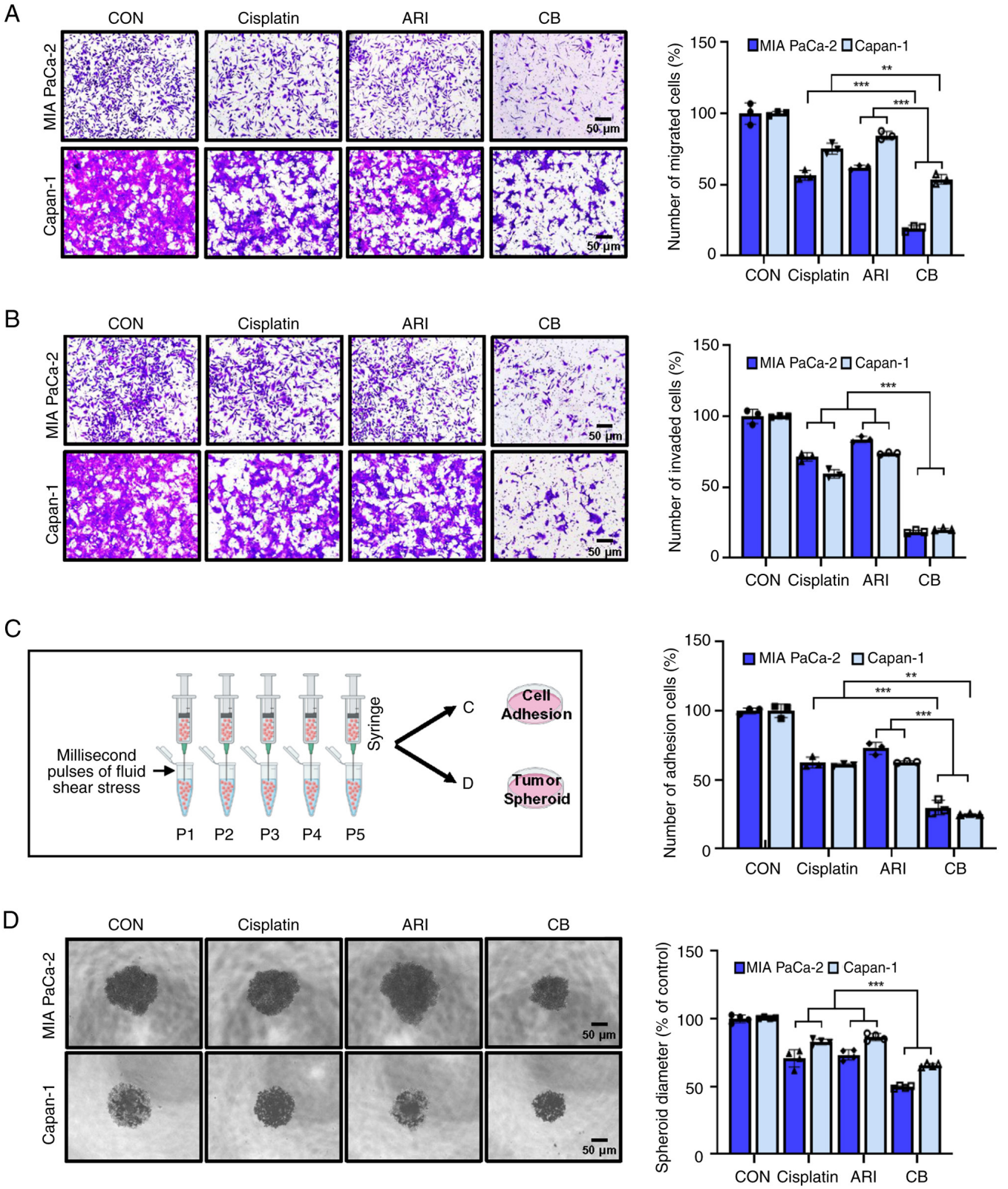

Figure 4. Inhibition of the metastasis by the combination of cisplatin and aripiprazole. (A and B) Transwell migration and invasion assays. (C) Scheme of the fluid shear stress assay for assessing cell adhesion after treatment. (D) Three-dimensional spheroid cluster assay demonstrating the effect of combination treatment of metastatic potential. All experiments were performed in three replicates. Data are presented as means ± standard deviation. **P<0.01 and ***P<0.001. CON, control; ARI, aripiprazole; CB, cisplatin and aripiprazole.

attachment to collagen-coated plates were significantly diminished by the combined treatment, in contrast to the effects of single-agent treatments (Fig. 4C and D).

Aripiprazole inhibits the STAT3 pathway. Among the 43 kinases analyzed, aripiprazole inhibited STAT3 expression by 40% (Fig. 5A). To validate these findings, cells were treated

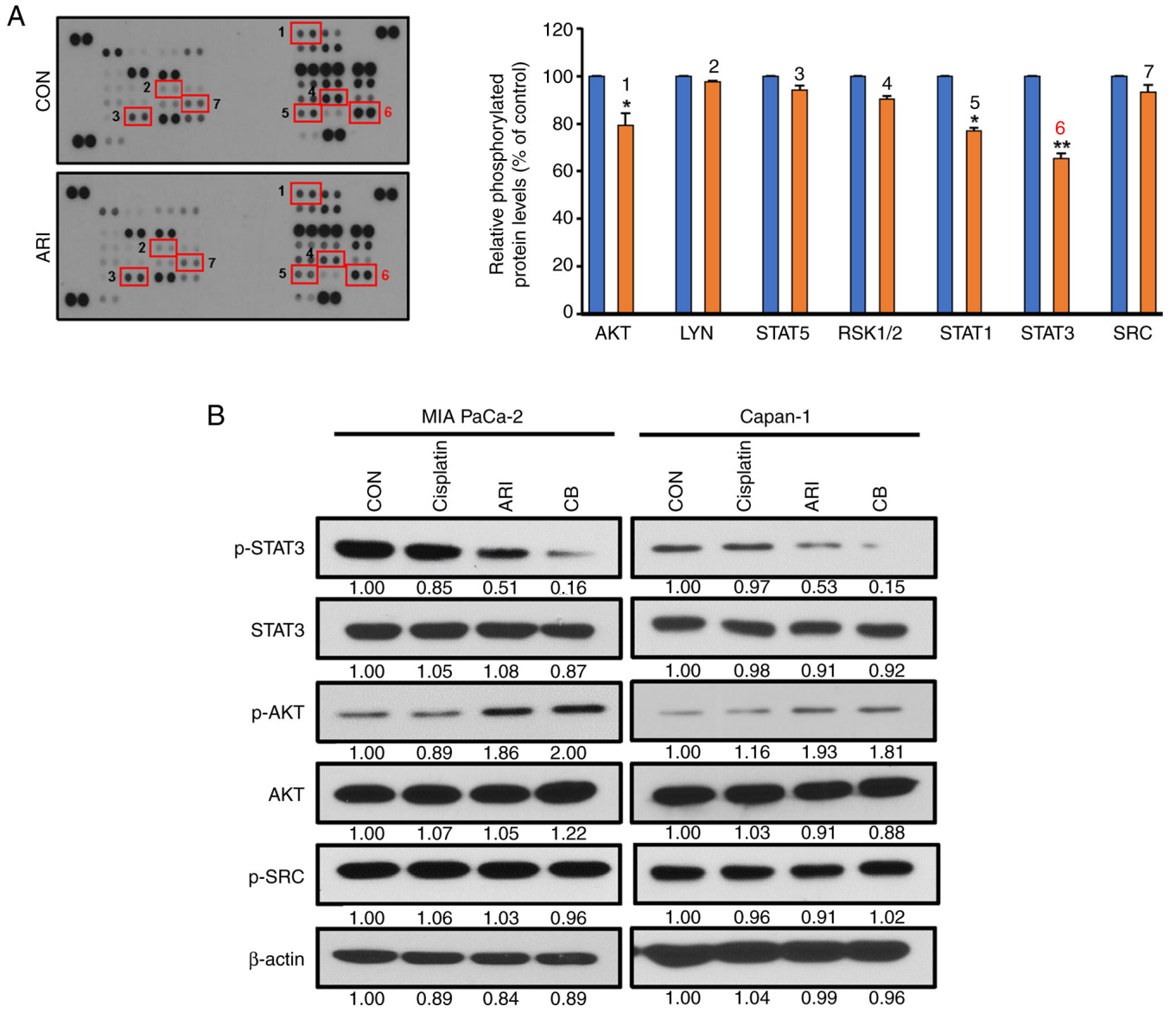

Figure 5. Inhibition of the STAT3 pathway by aripiprazole. (A) Phospho-kinase array analysis of MIA PaCa-2 cells treated with aripiprazole for 12 h, showing levels of phosphorylated kinases. (B) Western blot analysis of p-AKT, p-SRC and p-STAT3 in pancreatic cancer cells treated with cisplatin and aripiprazole. Data are presented as means ± standard deviation. *P<0.05 and **P<0.01. p-, phosphorylated; CON, control; ARI, aripiprazole; CB, cisplatin and aripiprazole.

with aripiprazole (10 μM) for 12 h, followed by western blot analysis to examine the expression of p-STAT3, p-AKT and p-SRC. As shown in Fig. 5B, aripiprazole decreased p-STAT3 expression, and the combination of aripiprazole and cisplatin almost completely abolished its expression in MIA PaCa-2 cells. These findings suggested that the combination treatment could induce pancreatic cancer cell death by inhibiting STAT3 signaling.

Aripiprazole inhibits tumor growth in mouse pancreatic cancer models. Tumor growth was delayed in mice treated with either aripiprazole or cisplatin alone compared with the control group. Notably, the combination treatment significantly reduced tumor volume by 78% relative to the control (Fig. 6A). These results were corroborated using an orthotopic pancreatic cancer model (Fig. 6B). All treatments were well-tolerated, with no significant differences between groups. Tumor formation incidence was >95%. Treatment with aripiprazole or cisplatin inhibited primary pancreatic tumor growth by 33 and 23%, respectively, compared with the control. The combination treatment markedly enhanced the anti-tumor effect, with a 73% growth inhibition observed compared with control. Furthermore, the combined treatment reduced the expression of Ki-67 and Bcl-2, markers of cell proliferation and apoptosis, respectively and increased the expression of cleaved caspase-3 and the number of TUNEL-positive cells. The combination treatment also significantly reduced p-STAT3 expression (Fig. 6C).

Discussion

Of patients with pancreatic cancer, ~80% are diagnosed at a locally advanced or metastatic stage, where combination chemotherapy is generally preferred over single-agent chemotherapy. However, the effectiveness of these treatments remains limited, prompting increased interest in combination

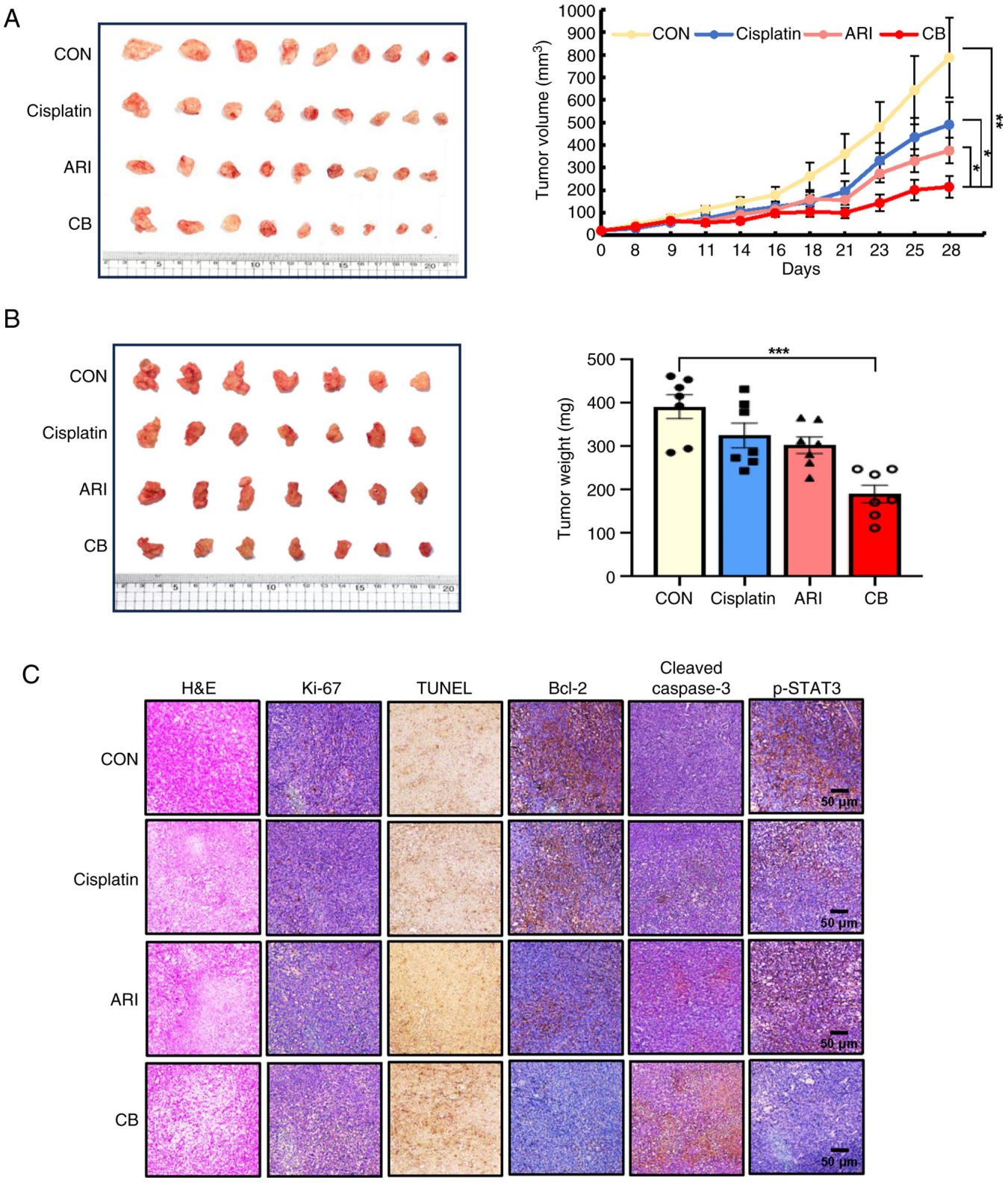

Figure 6. Antitumor effects of combined cisplatin and aripiprazole treatment in pancreatic cancer mouse models. (A) Tumor weights in MIA PaCa-2 xenografts after 28 days of cisplatin (2 mg/kg) and aripiprazole (10 mg/kg) co-treatment (n=9 per group). (B) Effects of cisplatin (2 mg/kg) and aripiprazole (10 mg/kg) co-treatment in KPC orthotopic mouse models after 25 days (n=7 per group). (C) Hematoxylin and eosin staining and immunohistochemical detection of Ki-67, TUNEL, Bcl-2, cleaved caspase-3 and p-STAT3. Data are presented as means ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; p-, phosphorylated; CON, control; ARI, aripiprazole; CB, cisplatin and aripiprazole.

therapies with fewer side effects and higher efficacy. Currently, cisplatin is used to treat various solid tumors, including PDAC. Despite its usefulness, cisplatin has shown severe side effects drug resistance, potentially contributing to treatment failure in PDAC (4,20). Nevertheless, cisplatin continues to be evaluated in clinical trials for its potential in combination chemotherapy

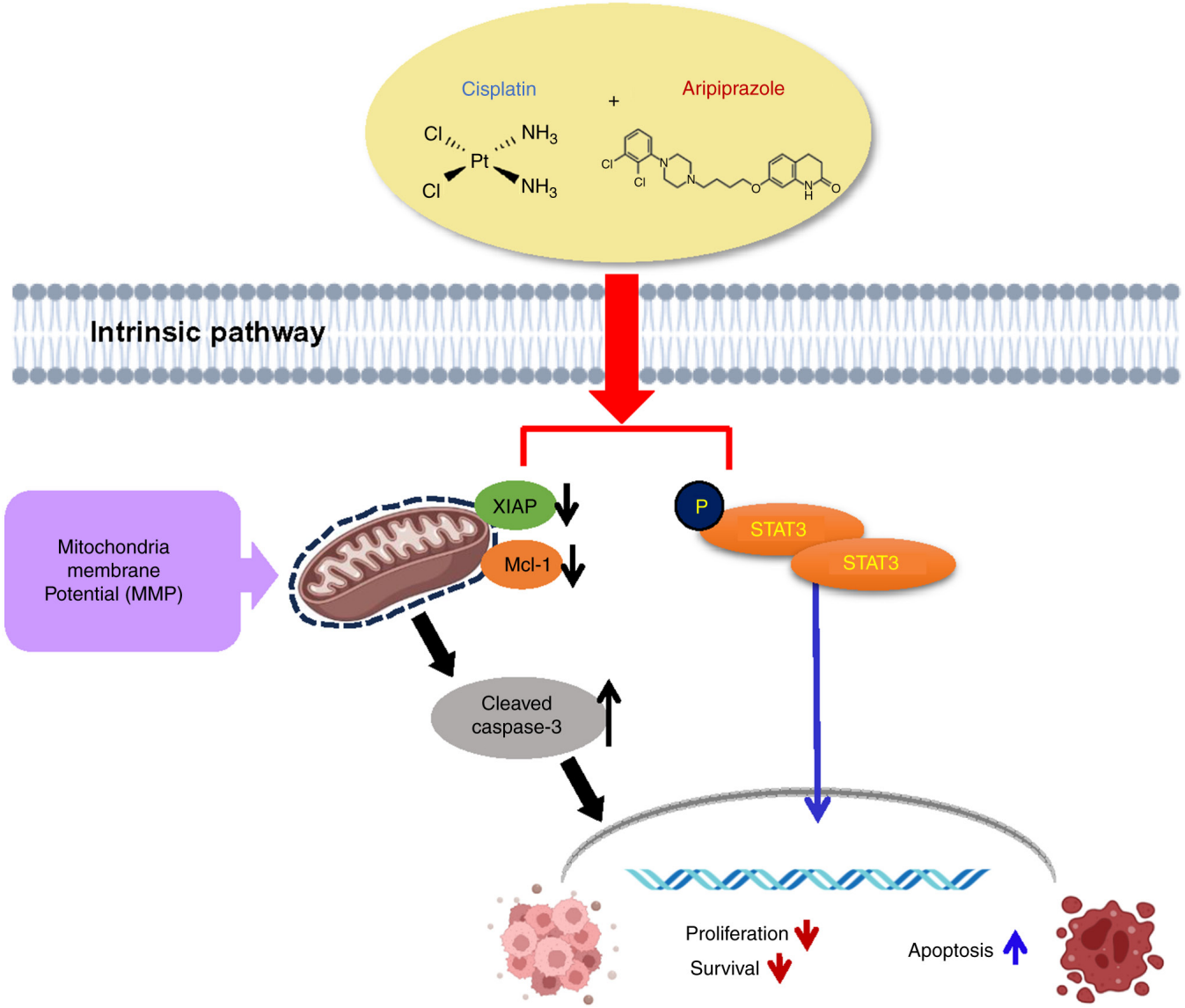

Figure 7. Schematic representation of how combined cisplatin and aripiprazole treatment leads to potent antitumor activity via the STAT3 signaling pathway. MCL-1, myeloid cell leukemia-1; XIAP, X-linked inhibitor of apoptosis protein.

for PDAC. Therefore, combination treatment with sensitizing agents that have fewer side effects may be an effective strategy for improving anticancer efficacy and overcoming cisplatin resistance. The present study found that the combination of aripiprazole and cisplatin synergistically induced apoptosis and inhibited cell growth by blocking STAT3 signaling. Moreover, this combination significantly reduced tumor growth in animal models without notable side effects, suggesting that the combination of aripiprazole and cisplatin could enhance PDAC treatment outcomes.

Aripiprazole, an atypical antipsychotic, which is effective in treating schizophrenia and schizoaffective disorders, exhibits unique pharmacological activities as a serotonin 5-HT1A and 5-HT2A antagonist. During tumor development, cancer not only evades the body's regulatory mechanisms, but also affects local and systemic homeostasis. It has been shown in human and animal cancer models that tumors affect the production of classic neurotransmitters such as hypothalamic and pituitary hormones (21). Indeed, the neurotransmitter regulator aripiprazole shows anti-cancer effects across different types of cancer cells (13,14). This drug repositioning strategy has recently demonstrated potent radiosensitizing activity in various cancer cells (18,22). Therefore, it was hypothesized that combining cisplatin with aripiprazole could result in synergistic effects in PDAC cells. The combined treatment significantly inhibited PDAC cell growth compared with individual agent treatments. Cotreatment with cisplatin and aripiprazole exhibited the highest synergistic effects in PDAC cells, as indicated by the CI values. The present study investigated whether this combination induced synergistic apoptotic effects. Indeed, it led to significant apoptosis, evidenced by increased TUNEL-positive nuclear fragmentation and elevated cleaved caspase-3 expression. While studies have confirmed the apoptotic effect of aripiprazole in combination with other antipsychotic drugs such as fluphenazine, halaven and thioridazine in cancer (14,18), few have explored its combination with anticancer drugs for clinical use. Therefore, the apoptotic effect observed with aripiprazole combined with cisplatin appears to be substantial.

Apoptosis occurs through two distinct pathways: The extrinsic pathway, activated by death receptors, and the intrinsic pathway, involving mitochondrial factors. Given reports that the combination of cisplatin with other agents induces the intrinsic mitochondrial apoptotic pathway (23,24), the present study extended its analysis to investigate whether aripiprazole affected mitochondrial membrane potential when combined with cisplatin, using JC-1 staining. As expected, the combined treatment synergistically reduced mitochondrial membrane potential. To validate these findings, the expression of XIAP and MCL-1, members of the inhibitor of apoptosis proteins (IAPs) family crucial in mitochondria-mediated apoptosis, were examined. The analyses demonstrated a significant reduction in XIAP and MCL-1 expression alongside increased levels of cleaved caspase-3 in PDAC cells following combined treatment. Overall, the results suggested that the synergistic apoptotic effects of this combination therapy may operate through the mitochondria-mediated apoptotic pathway in PDAC cells, potentially contributing to the inhibition of tumor growth in animal models.

To date, aripiprazole has been reported to modulate the cAMP/PKA, ERK/C-Fos and AKT/GSK3β signaling pathways in brain diseases (14,25,26). Studies have indicated that aripiprazole enhances cancer cell sensitivity to ionizing radiation by increasing reactive oxygen species production (17) and that SRC serves as a primary target for aripiprazole's anti-tumor activity in glioma cells (13). However, there has been limited research exploring the precise mechanisms by which aripiprazole affects cancer signaling pathways. To further investigate the potential mechanism underlying the anticancer effects of aripiprazole, a phosphokinase array analysis was conducted. The present study demonstrated that aripiprazole effectively inhibited STAT3 phosphorylation. Notably, contrary to previous reports (13,27), increased expression of AKT and SRC was observed in response to aripiprazole in pancreatic cancer cells, suggesting differential effects on AKT and SRC signaling pathways. Given that STAT3 is frequently overexpressed in tumors and its increased signaling promotes cancer cell survival and chemoresistance, the inhibition of p-STAT3 expression by the combination of aripiprazole and cisplatin, mediated by aripiprazole, holds significant therapeutic promise.

In conclusion, the present study is the first, to the best of the authors' knowledge, to demonstrate that combined treatment of aripiprazole and cisplatin markedly inhibited PDAC cell growth and synergistically exhibited anticancer activities by suppressing cell proliferation and inducing apoptosis both *in vitro* and *in vivo* via STAT3 pathway inhibition (Fig. 7). Aripiprazole is an FDA approved antidepressant currently in clinical use; therefore, it is expected to be useful as an excellent adjuvant that can markedly increase the efficacy of cisplatin therapy for pancreatic cancer. These findings suggested that the combination of aripiprazole and cisplatin represents an innovative therapeutic approach for pancreatic cancer in humans.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YJC performed all the experiments with the assistance of BSH, SK, MSP, YJL, SEK, PL and HGG. HL, SK, SP and ERP interpreted the results. YJC, KHJ and SH wrote the manuscript. SSH and KHJ contributed to the design of the study and assembled data. SSH and KHJ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal protocols were approved by the INHA Institutional Animal Care and Use Committee (approval no. INHA IACUC) at the College of Medicine, Inha University (Incheon, Korea; approval nos. INHA 2211124-848 and INHA 230731-883).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, the authors used DeepL (www.deepl.com) to assist in translating the original text into English to check for grammatical errors and confirm alternative expressions. After using this tool, the authors reviewed and edited the content as necessary and therefore they take full responsibility for the ultimate content of the present manuscript.

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