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Sequence of CX-4945 and Cisplatin Administration Determines the Effectiveness of Drug Combination and Cellular Response in Cholangiocarcinoma Cells *In Vitro*

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Abstract. *Background: The incidence of cholangiocarcinoma (CCA) is increasing worldwide and current single chemotherapeutic drug treatments are ineffective. CX-4945 and cisplatin are currently in clinical trial for CCA treatment. Materials and Methods: We assessed the effects of the sequence of administration of CX-4945 and cisplatin applied in combination treatments on their efficacy in CCA cells in vitro. CCA cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Apoptosis was examined using flow cytometry. The percentage of cells positive for phosphorylated H2A histone family member X (γ-H2AX) were measured using both flow cytometry and immunofluorescence. Results: CCA cell viability was reduced to 50% after 24 h of treatments with CX-4945 and cisplatin as single agents. Interestingly, treatment with cisplatin 6 h prior to CX-4945 treatment induced significantly more DNA damage and apoptosis than CX-4945 treatment followed by cisplatin. Unexpectedly, CX-4945 treatment followed by cisplatin was less effective than single treatment in RMCCA-1 CCA cells. In addition, a 1:1 ratio of each drug was the most effective combination in these cells. Conclusion: These data demonstrate that the combination of CX-4945 and cis platin acts additively when cisplatin is applied first, at least in part due to increased DNA damage and apoptosis. Furthermore, treatment with CX-4945 prior to cisplatin treatment reduces the efficacy of this drug combination in CCA cells.*

Cholangiocarcinoma (CCA) is a lethal cancer of bile duct epithelial cells that is not responsive to single chemotherapeutic drugs (1). CCA is a very heterogenous disease that is resistant to nucleotide-based (2-6) and platinum-based (2) drugs. Incidence and mortality rates are increasing, and the disease is usually advanced at presentation. Median survival in the UK is very poor; only 30% of patients are suitable for surgical resection and 5-year survival for these patients is less than 50%. A worse trend is observed in Thailand, where only 2% of patients are suitable for surgical resection (7) and 5-year survival is only 22%, 14%, 9% and 7% for patients aged 30-40, 41-45, 51-60 and 61-98 years, respectively (8). Advanced or metastatic disease has a very poor prognosis, with median overall survival of 12.2 months in the UK and 53 days in Thailand (7, 9). CCA is more prevalent in South-East Asia than in the UK due to a high incidence of liver fluke infection and exposure to chemicals in agricultural areas (10, 11), accounting for more than 15,000 deaths/year in Thailand alone.

Protein kinase CK2 (formerly known as casein kinase 2) is a potential drug target for CCA (12). CK2 protein levels or kinase activity are increased in many cancer cell types, including CCA (12). Inhibition of CK2 or gene disruption/knockdown in CCA cells results in increased apoptosis (13, 14). Importantly, CCA cells with reduced CK2 activity appear to be more sensitive to the chemotherapeutic drugs 5-fluoruracil and gemcitabine (12). The synthetic CK2 inhibitor, CX-4945, was shown to induce apoptosis and methuosis in CCA tumor cells *in vitro* (15) and inhibit the proliferation of CCA tumor cells *in vitro* and *in vivo* (14), with increased apoptosis in primary CCA tumors (13). Therefore, CX-4945 was recently introduced into a phase I/II clinical trial for patients with CCA (ClinicalTrials.gov identifier:

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NCT02128282). The trial proposes combining CX-4945 with gemcitabine and cisplatin. A preclinical study showed that this drug combination inhibited the growth of CCA cell lines more effectively than treatment with a single drug (14).

We reported that CX-4945 can induce methuosis *in vitro* in CCA, breast cancer and prostate cancer cell lines, and in some immortalized normal epithelial cell lines *via* a protein kinase CK2-independent mechanism (15). Methuosis is a non-canonical form of cell death characterized by the formation of cytoplasmic vacuoles from distinctive macropinocytosis (16). The combination of CX-4945 with cisplatin, where the former can induce methuosis as early as 4 h after treatment (15) and apoptosis at 48 h (14), whilst the latter induced apoptosis (17, 18), triggered two distinct cell death mechanisms, and affected the efficacy of the combination.

Materials and Methods

Cell culture. The RMCCA-1 cell line (19) was established from a peripheral CCA specimen surgically obtained from a male Thai patient and was kindly provided by Associate Profesor Rutaiwan Tohtong at Mahidol University. RMCCA-1 cells passage between 24-36 were used. CCLP-1 was established from an intrahepatic CCA specimen from a female Caucasian patient (20) and was kindly provided by Dr. Simon Afford at the University of Birmingham. CCLP-1 cells passage between 20-38 were used. All cell lines were maintained in Dulbecco's modified Eagle's medium (HAD3024302, Hyclone, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% non-essential amino acid (Gibco) and 1% penicillin/streptomycin (Gibco). All cell lines were maintained at 37˚C with 5% CO_2.

Drug treatment. CX-4945 and cisplatin were added to CCA cells either simultaneously, or CX-4945 was followed by cisplatin or cisplatin was followed by CX-4945. The first drug in sequential treatment was given 6 h prior to the second drug, as illustrated in Figure 1.

Cell viability assay. Cells were plated and allowed to adhere overnight to achieve 80% confluency. They were then incubated with 1 to 100 μM in a half-log increment (1, 3.16, 10, 31.62 and 100 μM) of CX-4945 (Abcam, Cambridge, MA, USA) and cis-diammineplatinum (II) dichloride (cisplatin) (Tokyo Chemical Industry, Tokyo, Japan) either in a single or combined treatment as shown in Figure 1. Cisplatin was dissolved in aqueous solution containing 0.9% NaCl to maintain its stability (21, 22), while CX-4945 was dissolved in dimethyl sulfoxide. Cells were incubated for 24 h either with CX-4945 or with cisplatin alone, or in simultaneous/sequential combined treatments to determine whether there was synergism. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MilliporeSigma, Burlington, MA, USA) was then added to the final concentration of 0.5 mg/ml and cells were then incubated for 2.5 h at 37˚C. A half volume of stop solution (10% sodium dodecyl sulfate in 50% dimethylformamide in water) was added and mixed thoroughly before reading the absorbance at 570 nm on Multimode Plate Reader Victor Nivo (Perkin Elmer, Waltham, MA, USA).

Cell death analysis. Cells were plated to reach 80% confluency after overnight incubation. Then cells were treated with 20 μM of CX-4945 or cisplatin or their combination for 24 h as depicted in Figure 1. Cells were then stained with Muse™ annexin V and dead cell kit (MilliporeSigma) according to the manufacturer's protocol. Samples were read using Muse™ cell analyzer (MilliporeSigma) to determine the percentage of apoptotic and dead cells in the cell population. Data were analyzed using Muse™ software version 1.4 (MilliporeSigma).

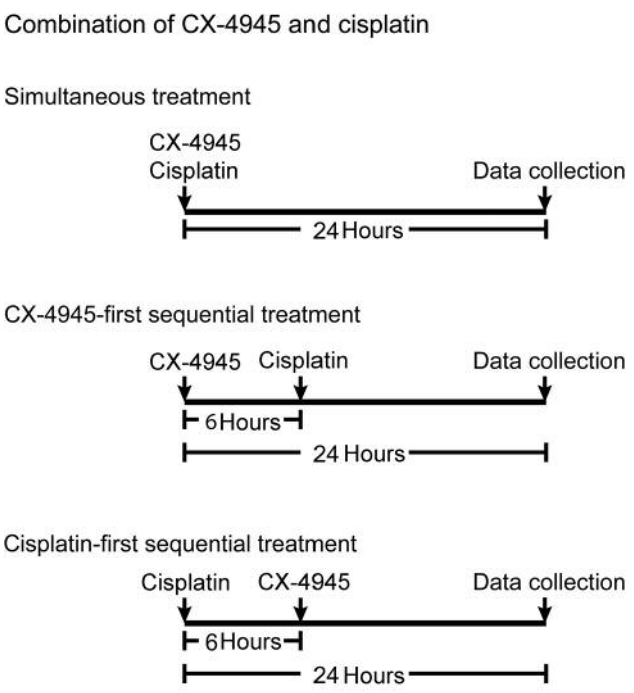

Figure 1. *Treatment scheme for single and sequential treatment with protein kinase CK2 inhibitor CX-4945 and cisplatin.*

Colony formation. Cells were plated at 500 cells per well. Sequential combination treatments, both cisplatin-first and CX-4945-first, were applied using a concentration of 20 μM. Cells were treated for 7 days before staining with 0.5% crystal violet solution in 12.5% glutaraldehyde in water for 15 min. Wells were washed with distilled water three times and air dried. Groups of 50 cells or more were considered as a colony and colonies were counted under inverted microscopy.

Flow cytometry. Cells were plated to reach 80% confluency after overnight incubation. The sequential treatments were applied to the cells for 24 h as depicted in Figure 1. CX-4945 and cisplatin were added at 20 μM. Cells were then trypsinized and washed with phosphate-buffered solution (PBS). Cells were permeabilized and fixed in 70% ethanol for 30 min at −20˚C then incubated in 1:100 γ-H2AX (Ser139) (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's recommendation, for 1 h at 23˚C. Cells were washed in PBS and incubated in 5 μg/ml goat anti-rabbit IgG (H+L) antibody conjugated to Alexa Fluor 488 (A-11008; ThermoFisher Scientific, Waltham, MA, USA) for 1 h at 23˚C. The percentage of γ-H2AX positive cells was assessed using a BD FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA,

USA) and data were analyzed with ModFit LT software version 3.0. Data were graphed relative to those of the vehicle control.

Immunofluorescent imaging. Cells were plated to reach 80% confluency after overnight incubation and then underwent sequential treatments for 24 h as depicted in Figure 1. Cells were trypsinized and washed with PBS, permeabilized and fixed in 70% ethanol for 30 min at −20˚C. Cells were incubated with primary antibodies against CK2α (1:100; Cell Signaling Technology) and γ-H2AX (Ser139) (1:100; Cell Signaling Technology) for 1 h at 23˚C. Cells were then washed in PBS and incubated in 5 μg/ml of secondary antibodies including goat anti-rabbit IgG (H+L) conjugated to Alexa Fluor 488 (ThermoFisher Scientific) and goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (ThermoFisher Scientific) for 1 h at 23˚C. Images were taken using the ImageXpress Micro XLS Widefield High Content Screening System (Molecular Devices, San Jose, CA, USA) under 20× magnification. The images were analyzed with MetaXpress analysis software version 6 under multiwavelength cell scoring module mode to obtain quantitative data.

Cell imaging. Live cell images were taken with a Nikon Eclipse T2S phase contrast inverted fluorescent microscope with 20× objective lens for vacuole counting. Vacuoles were counted manually.

Calculation of the half-maximal inhibitory concentration (IC_{50}) and 20 percent inhibitory concentration (IC_{20}). The IC_{50} of the single treatment was calculated using the AAT Bioquest online tool accessible at https://www.aatbio.com/tools/ic50-calculator. The online tool applies the following equation:

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + (\frac{X}{IC_{50}})^{Hill\ coefficient}}$$

Where Y is the cell number under treatment as a percentage relative to the vehicle control, X is the concentration of agent, Min is the minimum X value and Max is the maximum X value.

The IC_{20} was calculated from the IC_{50} using the GraphPad web tool available at https://www.graphpad.com/quickcalcs/Ecanything1.cfm. The online tool applies the following equation:

$$IC_F = \left[\frac{F}{100 - F}\right]^{1/H} \times IC_{50}$$

Where F is percentage inhibition (20 for IC_{20}) and H is the Hill slope.

Synergism analysis. Synergism was calculated using the combination index (CI) according to Chou (23). The following formula was used.

$$\text{CI}(f(a)) = \frac{X_a^c(f(a))}{X_a^o(f(a))} + \frac{X_b^c(f(a))}{X_b^o(f(a))}$$

Where $f(\alpha)$ is the determined degree of inhibition, X^o_a is the concentration of drug A alone to achieve the determined degree of inhibition, X^c_a is the concentration of drug A with the presence of drug B to achieve the determined degree of inhibition, X^o_b is the concentration of drug B alone to achieve the determined degree of inhibition, X^c_b is the concentration of drug B with the presence of drug A to achieve the determined degree of inhibition.

The CI is used to determine the synergism of the combination where a CI <1 means the combination effect is synergistic, a CI of 1 means the combination effect is additive, and a CI >1 means the combination effect is antagonistic.

Statistical analysis. Data were graphed as the mean±standard deviation. Statistical analyses were performed using analysis of variance with Dunnett's test unless otherwise stated. All experiments were performed at least in triplicate.

Results

Cisplatin and CX-4945 reduced CCA cell viability in a dose-dependent manner. Both cisplatin and CX-4945 reduced the viability of RMCCA-1 and CCLP-1 cell lines. Cisplatin at 30 μM reduced cell viability to 71.4% and 76.7%, respectively, while 100 μM cisplatin further reduced cell viability to 43.3% and 29.2%, respectively (Figure 2A). In addition, CX-4945 demonstrated a similar trend for both cell lines, reducing cell viability to 75.3% and 75.2%, respectively, at 30 μM. RMCCA-1 was less responsive to 100 μM CX-4945 as compared to CCLP-1, with cell viability of 61.8% compared with 51.2%, respectively (Figure 2B). The IC_{50} for 24-h cisplatin treatment on RMCCA-1 cells was 76.2 μM and that for CCLP-1 cells was 68.7 μM. The IC_{50} for 24-h CX-4945 treatment for CCLP cells was 109.3 μM, while a concentration of CX-4945 as high as 100 μM inhibited RMCCA-1 cells by less than 50%.

The sequence of CX-4945 and cisplatin treatment determined the efficacy of the combination. To determine the effect of the sequence of drug addition, CCA cells were treated with CX-4945 or cisplatin alone for 24 h, or CX-4945 or cisplatin for 6 h before the addition of the second drug for a further 18 h as demonstrated in Figure 1. Drugs were used at the IC_{20} values as shown in Table I.

Our previous studies showed that CX-4945 treatment induced methuosis in CCA cell lines 4 h after treatment and in the absence of significantly increased apoptosis at this time point (15). In this study, simultaneous treatment divided cells into two populations, one with cytoplasmic vacuoles and another with no vacuoles (Figure 3A and B). Notably, sequential treatment, particularly cisplatin-first, did not induce vacuoles in the cytoplasm (Figure 3C and D). Therefore, we further investigated the effect of sequential treatment.

Muse™ annexin V and dead cell assay was used to measure the percentage of cells in early and late apoptosis and dead cells. Interestingly, different responses for apoptosis induction of the cells receiving simultaneous treatment were seen as indicated by the large standard deviation (Figure 4A).

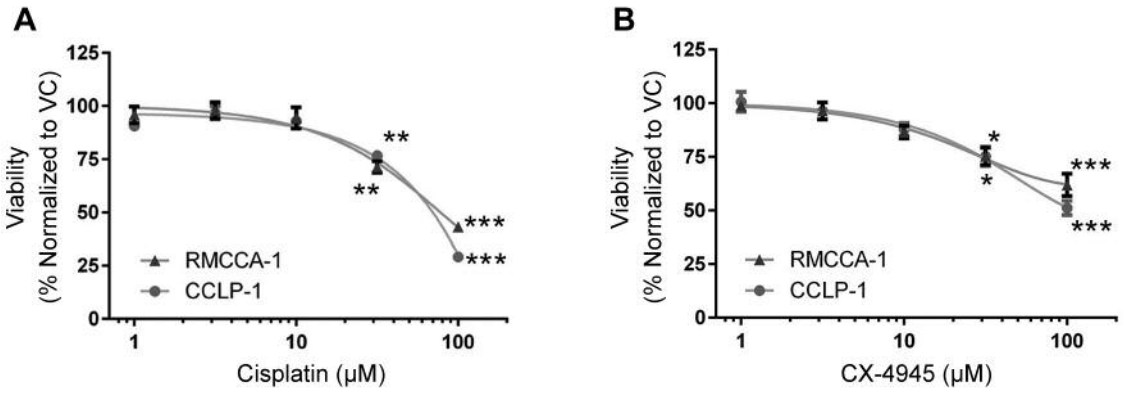

Figure 2. *Protein kinase CK2 inhibitor CX-4945 and cisplatin reduced cholangiocarcinoma cell viability. Cisplatin (A) and CX-4945 (B) reduced CCA cell viability in a dose-dependent manner as determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assays. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as mean±SD. VC: Vehicle control. Significantly different at: *p<0.05, **p<0.01 and ***p<0.001.*

CX-4945-first treatment did not induce significantly higher levels of apoptosis compared to treatment with each drug alone (Figure 4B). In contrast, cisplatin-first treatment resulted in an increase in the number of apoptotic cells (Figure 4B). CX-4945-first treatment induced apoptosis of 27% in RMCCA-1 and 37% in CCLP-1 cells, while CX-4945-first treatment induced apoptosis of only 14% and 19%, respectively (Figure 4B). CX-4945-first treatment was less effective than both single drugs in RMCCA-1 cells (Figure 4B). Notably, the efficacy of cisplatin-first treatment on apoptosis induction was 95% and 88% more effective in RMCCA-1 and CCLP-1 cells, respectively, as compared to CX-4945-first treatment (Figure 4C).

To confirm that the difference in efficacy observed between cisplatin-first and CX-4945-first treatments was not simply a short-term effect, we utilized colony formation to study the long-term effect of these combinations. Cisplatin-first treatment was more effective than CX-4945-first treatment in this assay. Cisplatin-first treatment reduced the number of colonies by two-thirds or more in RMCCA-1 and CCLP-1 cells (Figure 5). In contrast, CX-4945-first treatment only reduced the number of colonies by approximately half (Figure 5).

Twenty micromolar concentration of cisplatin and CX-4945 was used in the previous experiments (Figure 3 and Figure 4), thereby the effects of a 1:1 ratio of the agents was examined since the drug ratio in the combination experiments above might have been critical for the efficacy of the combination. Further investigation was performed to optimize the ratio of the combination by using 1:1, 1:2, 1:3 and 1:4 ratios and *vice versa*. None of the ratios tested led to significantly different results at the high doses in RMCCA-1 (10 μM and 31.62 μM) and CCLP-1 (31.62 μM) cells (Figure 6A). All the drug ratios tested inhibited CCA cell viability by approximately 40% and 30% in RMCCA-1 and CCLP-1 cells, respectively, when treated with the highest dose of the combination. To further examine the combination efficacy of different drug ratios, we compared the combinations with the single drugs at the same concentration. We found that the 1:1 ratio was the most effective because this ratio had the highest additive effect (as indicated by Δ) when compared at 31.62 μM, which is the highest dose (Figure 6B and C).

Table I. *Concentration of CX-4945 and cisplatin required for the inhibition of 20% of cells (IC_{20}) in two cholangiocarcinoma cell lines.*

	Cell line	
IC_{20}	RMCCA-1	CCLP-1
Cisplatin	20.6 μM	25.9 μM
CX-4945	17.4 μM	28.7 μM

IC_{20} values were calculated using web calculator available at: https://www.graphpad.com/quickcalcs/Ecanything1.cfm

To clearly see the combination effect of these drugs at 31.62 μM, which is the concentration that showed the highest additive effect, data from use of all ratios of 31.62 μM combination from Figure 6B and 6C are presented in a bar graph (Figure 7).

The 1:1 drug ratio showed the highest increase in inhibitory effect (Figure 7A and B). However, none of these combination ratios show significant synergistic effect. The ratios of 1:1 in RMCCA-1 cells, and 1:1 and 1:2 in CCLP-1 cells showed marginal synergistic effect. Combination ratio of 1:1 in RMCCA-1 cells inhibited cell viability by 57.8%, while the sum of inhibition from single drugs was 52.3%

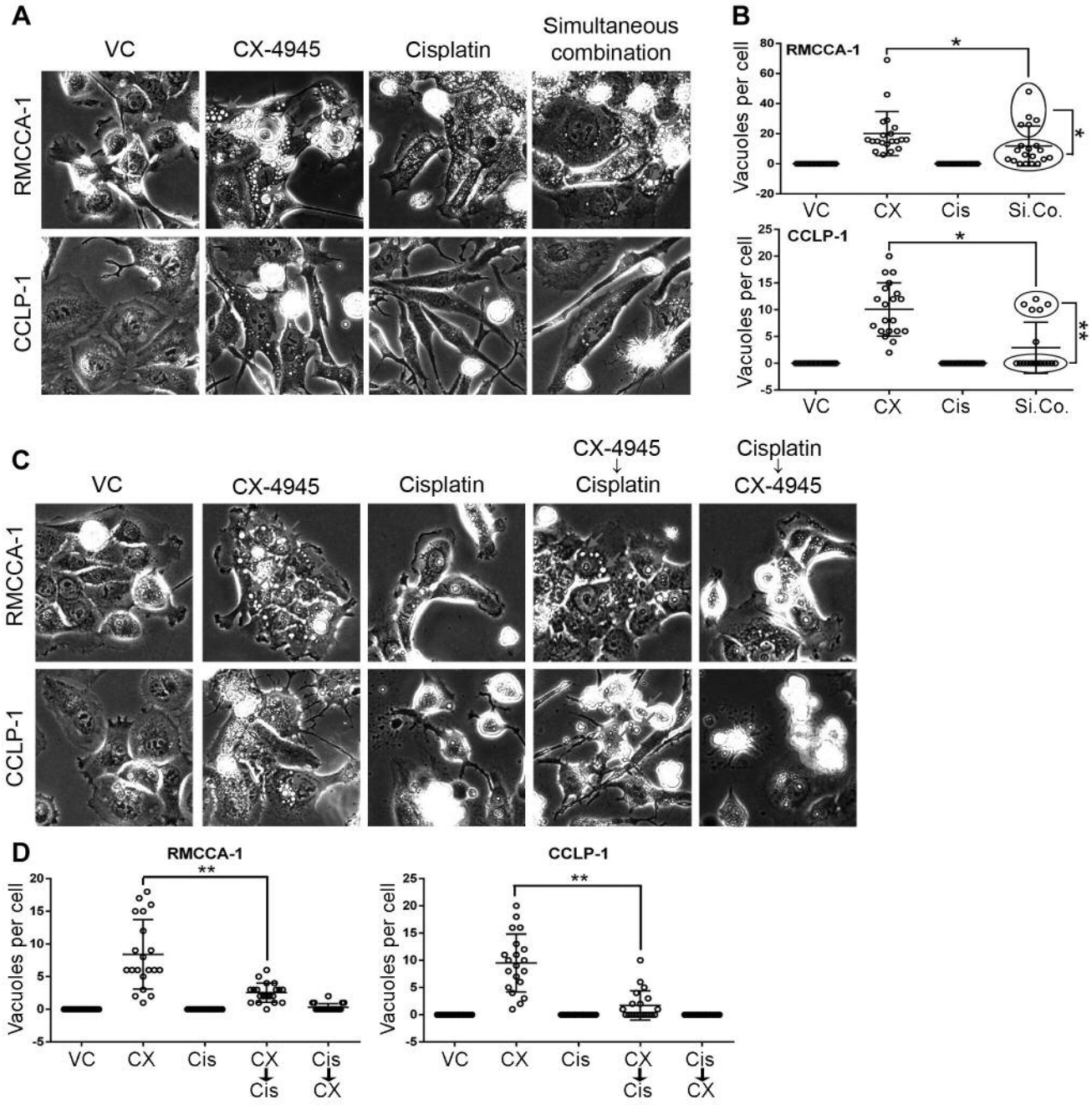

Figure 3. *Different populations of cholangiocarcinoma (CCA) cells respond to simultaneous treatment with CX4945 and cisplatin differently and cisplatin reduced CX-4945-induced methuosis. The number of vacuoles in CCA cells treated with each drug alone and in combination or sequentially was determined. A, B: CX-4945 at 10 μM induced cytoplasmic vacuoles, while 30 μM cisplatin did not. The combination of CX-4945 and cisplatin reduced cytoplasmic vacuolization in all both CCA cell lines. C, D: Arrows indicate cytoplasmic vacuoles. VC: vehicle control. Original magnification, 200×. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as the mean±SD. Significantly different at: *p<0.05 and **p<0.01.*

(27.6% from cisplatin + 24.7% from CX-4945) (Figure 7A). Similarly in CCLP-1 cells, where the 1:1 ratio inhibited cell viability by 58.3%, the sum of inhibition from the single drugs was 48.1% (23.3% from cisplatin + 24.8% from CX-4945) (Figure 7B). The ratio of 1:2 in CCLP-1 cells also gave a non-synergistic effect, with the combination inhibiting cell viability by 62.7% and the sum of single drugs by 58.3%. Other ratios in both cell lines gave less inhibitory

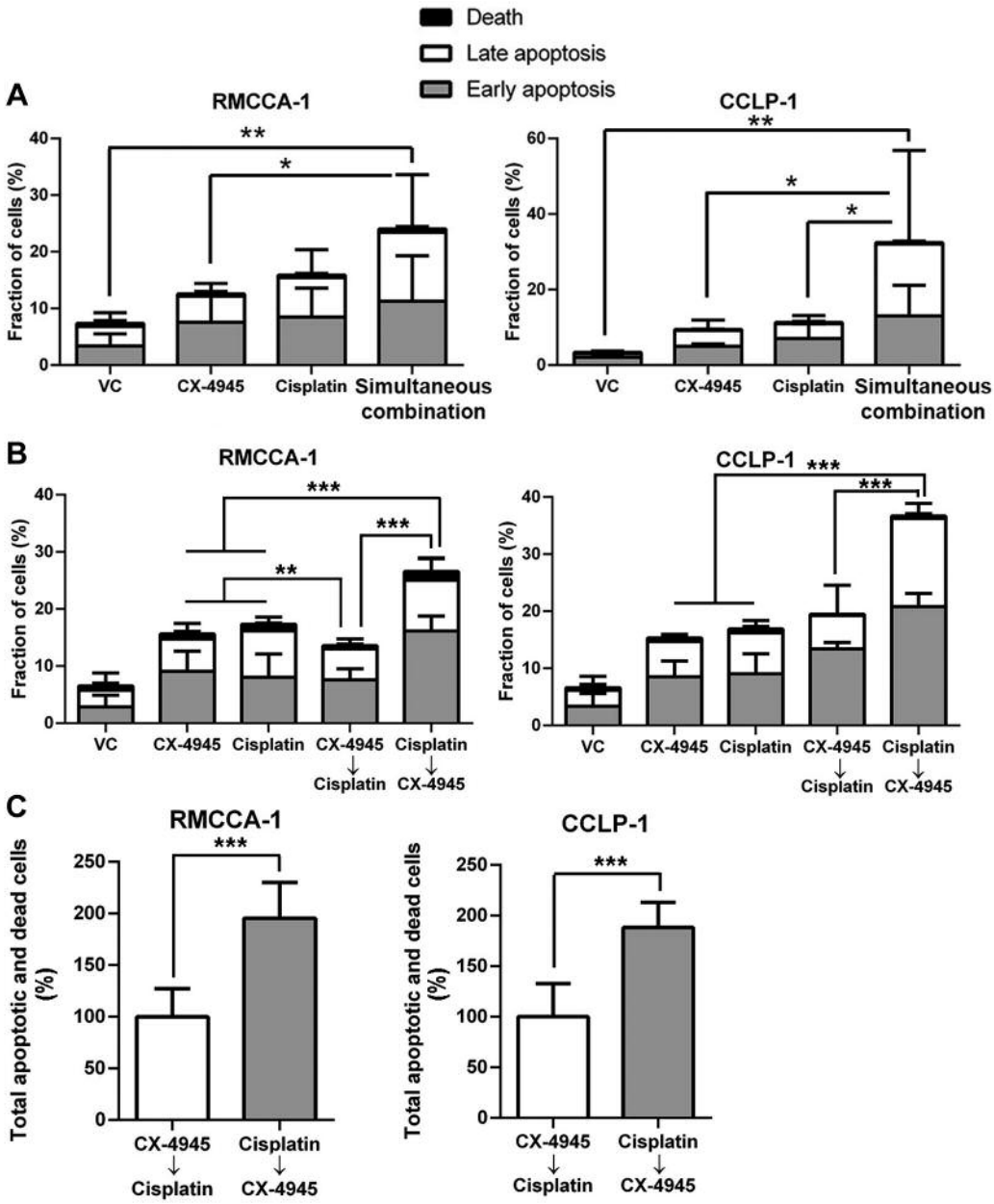

Figure 4. *Treatment with a combination of cisplatin and CX-4945 induced apoptosis in cholangiocarcinoma cell lines and the sequence of the combination determined the efficacy of the combination. A: Simultaneous combination was more effective than single drugs. B: Cisplatin-first treatment induced more apoptotic cells in both RMCCA-1 and CCLP-1 cell lines as compared to both drugs alone and CX-4945-first treatment. C: Cisplatin-first treatment was more effective than CX-4945-first treatment in apoptosis induction. Cisplatin and CX-4945 were used at 20 μM in these experiments. All experiments were performed in triplicate and with at least three biological replicates. Data were plotted as the mean±SD. VC: Vehicle control. Significantly different at: *p<0.05, **p<0.01, ***p<0.001.*

effect when comparing the combination treatment to the sum of effects from each drug singly.

To further determine the synergism of this combination, the CIs were calculated from the IC_{20} shown in Table II. CI values lower than 1 indicate a synergistic effect of the combination. Combination ratios of 1:1, 1:2, 1:4 and 2:1 cisplatin:CX-4945 against RMCCA-1 cells and all ratios in CCLP-1 cells led to CI values lower than 1 (Table II). Since the CI value was only slightly lower than 1.0, and the actual combination at the concentration that gave the highest

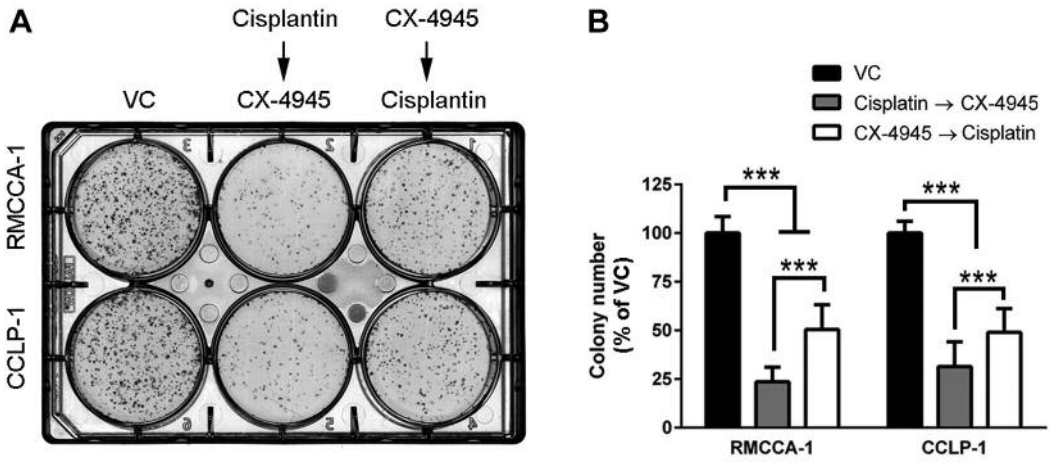

Figure 5. *Cisplatin-first treatment was more effective than CX-4945-first treatment in inhibition of colony formation by cholangiocarcinoma cells. A: Images of colonies formed by cells treated with vehicle control (VC), cisplatin-first or CX-4945-first combination treatments. B: The number of colonies in cells under cisplatin-first treatment was lower than in CX-4945-first-treated cells. Cisplatin and CX-4945 were both used in these experiments at 20 μM. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as the mean±SD. VC: Vehicle control. ***Significantly different at p<0.001.*

Table II. *Combination index (CI) of the treatment of cholangiocarcinoma cell lines. The CI was calculated according to Chou (23). CI values of <1, 1 and >1 indicate synergistic effect, combined effect and antagonism, respectively.*

Cell line	CI of treatment with cisplatin:CX-4945 at a ratio of						
	1:1	1:2	1:3	1:4	2:1	3:1	4:1
RMCCA-1	0.7	0.9	1.0	0.7	0.8	1.2	1.5
CCLP-1	0.7	0.5	0.7	0.8	0.7	0.8	0.8

additive effect (31.62 μM) demonstrated only an additive effect, we conclude that the combination of CX-4945 and cisplatin when giving cisplatin first had an additive effect on RMCCA-1 and CCLP-1 cells.

Cisplatin followed by CX-4945 treatment induced higher levels of DNA damage. Cisplatin is a DNA-damaging drug and CX-4945 inhibits protein kinase CK2 (15), a kinase that is directly involved in DNA repair (24). To examine the underlying mechanism behind the higher levels of apoptosis in cells treated with cisplatin first, we measured the percentage of cells positive for phosphorylated γ-H2AX, which is a marker for the DNA damage response (25-27). Cisplatin-first treatment induced 5.5- to 8-fold increase of γ-H2AX-positive RMCCA-1 and CCLP-1 cells, respectively, as compared to the vehicle control group (Figure 8A and B). CX-4945-first treatment induced a level of γ-H2AX positivity below that of cells treated with cisplatin first (Figure 8A and B). Cisplatin-first treatment was significantly more effective in inducing DNA damage than CX-4945-first treatment in both RMCCA-1 and CCLP-1 cell lines, as indicated by the higher level of γ-H2AX positivity (Figure 8C).

We further assessed the level of DNA damage using immunofluorescent microscopy to confirm the increase in γ-H2AX positivity, demonstrating it to be higher in the cisplatin-first treatment of both cell lines (Figure 9A). The number of γ-H2AX foci was used as an indicator of the number of double-strand DNA breaks (27). The number of γ-H2AX foci in both sequential treatments was compared to that of the vehicle control group (Figure 9B). Cisplatin-first treatment induced formation of more γ-H2AX foci than CX-4945-first treatment. The intensity of the fluorescent signal from γ-H2AX was also assessed. The intensity of γ-H2AX in cisplatin-first-treated cells more than doubled in both cell lines as compared to the vehicle control group (Figure 9C). As expected, CX-4945-first treatment induced lower levels of γ-H2AX expression in both cell lines (Figure 9C). These data demonstrate that cisplatin-first treatment was 54% and 72% more effective than CX-4945-first treatment in inducing γ-H2AX expression in RMCCA-1 and CCLP-1 cells, respectively (Figure 9D). This suggests that γ-H2AX expression is higher following cisplatin-first treatment and that double-strand DNA breaks are repaired less efficiently with this treatment.

Discussion

The established standard systemic chemotherapy for patients with CCA includes gemcitabine and cisplatin as the first-line

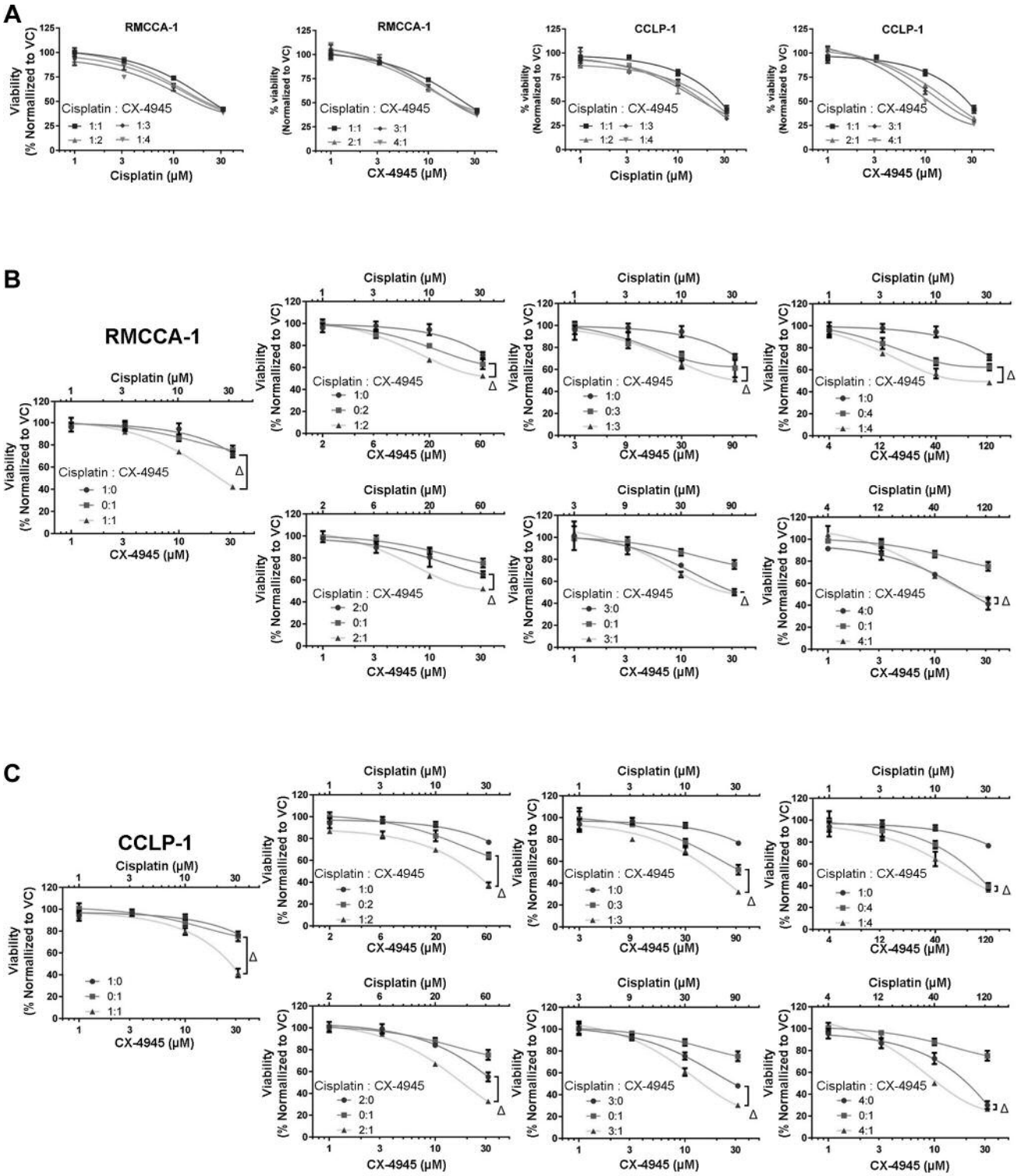

Figure 6. *The effect of different ratios of cisplatin to CX-4945 in cisplatin-first treatments on cholangiocarcinoma cell viability. Using different ratios of cisplatin and CX-4945 in cisplatin-first treatment of RMCCA-1 and CCLP-1 cells led to no significant difference in cell viability (A). A ratio of 1:1 (31.62 μM) cisplatin:CX-4945 showed the highest additive inhibition as compared to single drug treatment in both RMCCA-1 (B) and CCLP-1 (C) cells. Combination indices for each ratio are presented in Table II. VC: Vehicle control, Δ: Inhibitory shift. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as the mean±SD.*

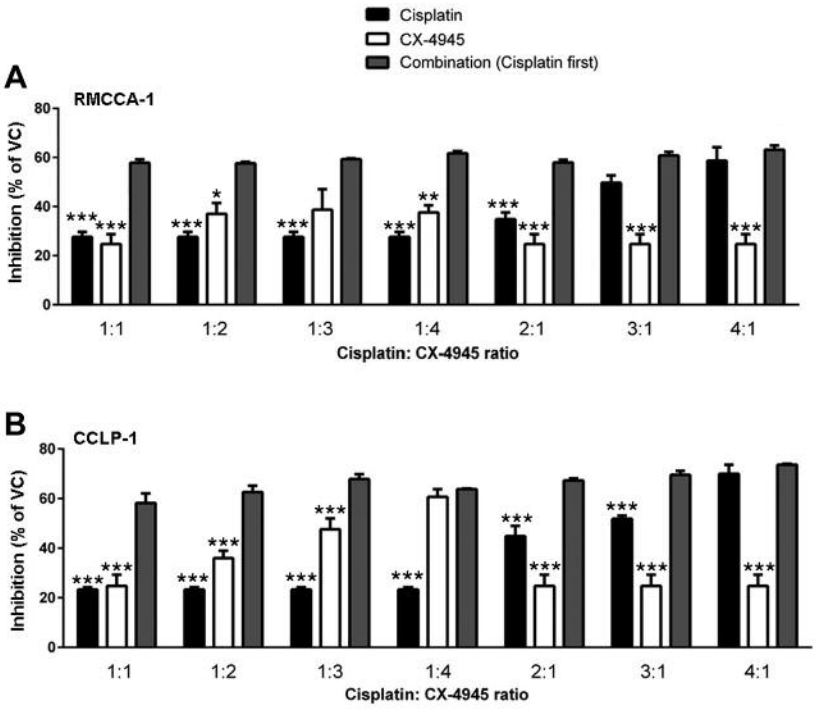

Figure 7. *Different combination ratios of cisplatin and CX-4945 were compared to single drugs at 31.62 μM (highest dose of the single drug used in this study) in RMCCA-1 (A) and CCLP-1 (B) cells. The 1:1 ratio of cisplatin and CX-4945 was the most effective against cholangiocarcinoma cells. VC: Vehicle control. Statistical analysis was performed to compare the effect of each single drug with that of the combination treatment. Note that the effects of combinations were not significantly different from the sum of the effects of the individual drugs. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as the mean±SD. Significantly different from the corresponding combination at: *p<0.05, **p<0.01 and ***p<0.001.*

treatment, while the second-line treatment is folinic acid, fluorouracil and oxaliplatin (28). The common adjuvant therapy for CCA is capecitabine (28). A combination of CX-4945 with cisplatin has been shown to reduce viability in CCA cell lines (14). Herein, we demonstrate that the sequence of CX-4945 and cisplatin treatment determines the efficacy of this combination on the induction of apoptosis and DNA damage in CCA cell lines.

Sequential administration of chemotherapeutic drugs is important to maximize the efficacy of the drug combination. Salvador-Barbero *et al*. recently demonstrated that sequential treatment with DNA-damaging chemotherapeutic drugs (taxol, nab-paclitaxel or paclitaxel) followed by the cyclin-dependent kinase 4/6 (CDK4/6) inhibitor (palbociclib) enhanced the efficacy of the combination in pancreatic ductal adenocarcinoma (PDAC) cells (29). Interestingly, the opposite sequence of this combination has been shown to have less efficacy than a DNA-damaging chemotherapeutic drug alone (29). Similarly, our study shows that in RMCCA-1 cells, CX-4945-first treatment is less effective than cisplatin-first treatment, and is even less effective than single drug treatment (Figure 3B). In addition, simultaneous addition of CDK4/6 inhibitors and gemcitabine or 5-fluorouracil gave variable effects on PDAC cells (30, 31). This combination resembles the simultaneous combination of CX-4945 and cisplatin reported here that has been shown either to be less effective or to have a highly variable effect when the less optimal sequential treatment is given (30-33).

We recently reported that CX-4945 can trigger a type of non-apoptotic cell death referred to as methuosis (15). This type of cell death features extensive cytoplasmic vacuole formation before death. Although CX-4945 was also shown to induce apoptosis (14), methuosis was shown to occur at an early time point (4 h after treatment) before apoptosis is detected (15). Little is known about the interaction between methuosis and apoptosis. No data have been reported on whether these two distinct types of cell death occur concurrently or are antagonistic. We demonstrated that the simultaneous addition of CX-4945 and cisplatin can trigger either methuosis or apoptosis in different subpopulations of CCA cells (Figure 4A and 4B). In simultaneous combination, we observed that some cells have no or a very small number of cytoplasmic vacuoles while some cells still form as many vacuoles as seen with CX-4945 treatment

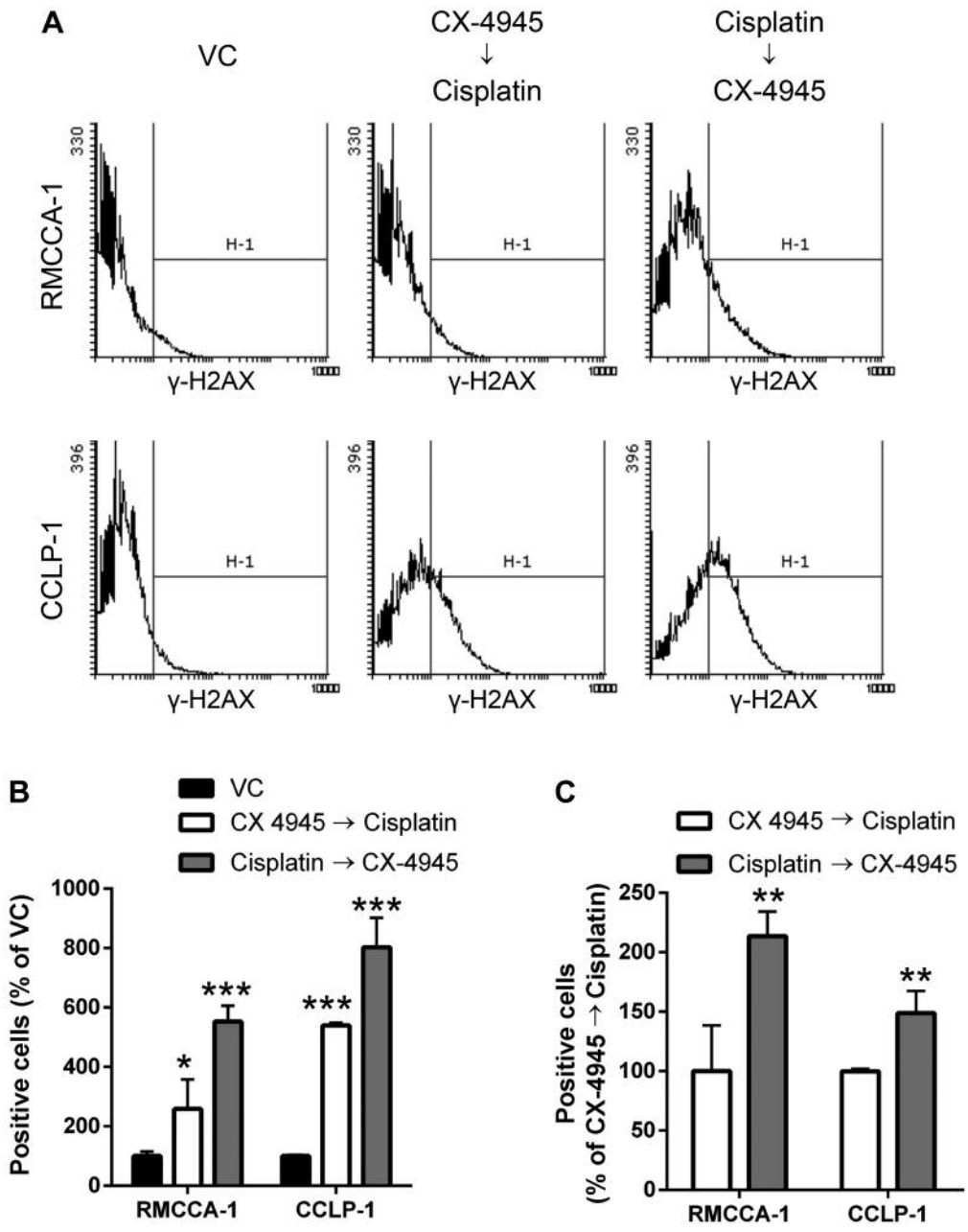

Figure 8. *Sequential treatment of cholangiocarcinoma cells with 20 μM cisplatin followed by 20 μM CX-4945 induced a higher proportion of cells positive for phosphorylated H2A histone family member X (γ-H2AX). A: Flow cytometry showed a greater proportion of cells positive for the DNA damage marker, γ-H2AX, with cisplatin-first treatment as compared to CX-4945-first treatment and the vehicle control group. B: Quantitative analysis of cytograms shown in A. C: Quantitative analysis of γ-H2AX-positive cholangiocarcinoma cells treated with cisplatin-first treatment relative to CX-4945-first treatment. VC: Vehicle control. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as the mean±SD. Significantly different at: *p<0.05, **p<0.01 and ***p<0.001.*

alone (Figure 3A and 3B). This resulted in a variable cellular response with a large standard deviation (shown in Figure 4A). This phenomenon might also be due to the fact that two distinct cell death mechanisms were induced in cells treated with the simultaneous cisplatin and CX-4945 combination (Figure 4). Interestingly, cisplatin reduced the CX-4945-induced formation of cytoplasmic vacuoles when added after CX-4945 and even prevented their formation

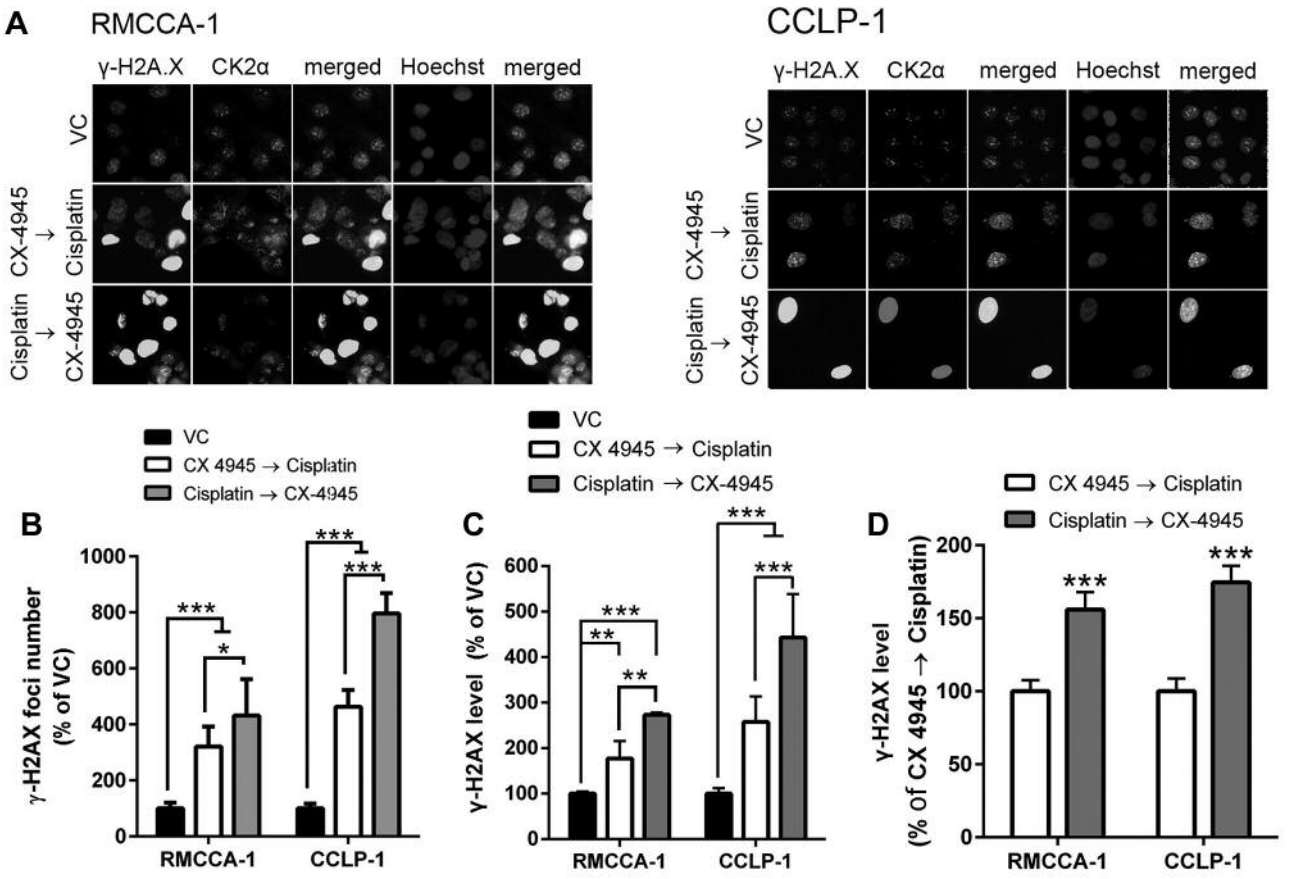

Figure 9. *Cisplatin followed by CX-4945 treatment induced more cell population with positive phosphorylated H2A histone family member X (γ-H2AX) in cholangiocarcinoma cells as compared to CX-4945-first treatment. A: Immunofluorescence staining showed higher cell population with positive γ-H2AX marker in cisplatin-first treatment as compared to CX-4945-first treatment. Original magnification, 40×. B: The number of γ-H2AX foci per cell in cells under cisplatin-first treatment was higher than that under CX-4945-first treatment and the vehicle control group. C: The γ-H2AX level measured using MetaXpress analysis software was higher in both sequential treatments as compared to the vehicle control group. D: Higher levels of γ-H2AX were present following cisplatin-first treatment as compared to CX-4945-first treatment. VC: Vehicle control. All experiments were performed in triplicate and with at least at three biological replicates. Data were plotted as the mean±SD. Significantly different at: *p<0.05, **p<0.01 and ***p<0.001.*

when used first (Figure 3C and 3D). Therefore, these data confirm that the sequence of drug addition determines CCA cellular response.

Similar to PDAC, the standard chemotherapeutic drugs for CCA are DNA-damaging drugs such as cisplatin and gemcitabine. It has been shown that the up-regulation of DNA repair in CCA cells after a DNA-damaging drug is administered is one of the common mechanisms of drug resistance (1). The effects of simultaneous addition of cisplatin and CX-4945 have been studied and reported to be additive on HuCCT1, EGI-1 and Liv27 CCA cell lines, which is due to the inhibition of DNA-repair mechanisms by CX-4945 (14). Herein, we further report that cisplatin-first treatment is more effective than CX-4945-first treatment and simultaneous treatment in RMCCA-1 and CCLP-1 CCA cell lines.

Similar to the administration of CDK4/6 inhibitors with DNA-damaging drugs in PDAC cells to achieve maximum efficacy, the administration of cisplatin (DNA-damaging drug) with CX-4945 should be sequential and in a specific order, namely cisplatin followed by CX-4945. This sequence was demonstrated here to cause higher DNA damage presumably due to less efficient DNA repair. This study provides critical information for a clinical trial. The current clinical trial on combining CX-4945 with gemcitabine and cisplatin (ClinicalTrials.gov identifier: NCT02128282) treats patients with either CX-4945 capsule on days 0, 1, 2, 7, 8, and 9 plus cisplatin infusion on days 1 and 8, or CX-4945 capsule for 10 consecutive days from days 0-9 plus cisplatin infusion on days 1 and 8. Based on our data, the treatment is likely be more effective when cisplatin is infused prior to CX-4945 administration.

Conclusion

The sequence of drug treatment is critical in using cisplatin and CX-4945 combination treatment for CCA. Efficacy of the combination *in vitro* was increased when cisplatin was used first followed by CX-4945. The 1:1 drug ratio was the most effective since it provided the greatest additive effect for this combination. Notably, the opposite sequence of addition was less effective than a single treatment. Therefore, it is likely that CX-4945 should be administered after cisplatin and the sequence of addition should be investigated in patients to determine whether the same effects are observed in the clinical setting.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization: J.L., K.G., P.-S.J. and J.S. Methodology: J.L., J.S., K.G. and P.-S.J. Validation: J.L., A.S., N.T, K.G., P.-S.J. and J.S. Formal analysis: J.L., A.S., and N.T. Investigation: J.L., A.S., and N.T. Resources, J.S., K.G. and P.-S.J. Writing-Original Draft Preparation: J.L. Writing-Review and Editing: J.L., J.S., K.G. and P.-S.J. Supervision J.S., K.G. and P.-S.J. Funding Acquisition: J.S., K.G., and P.-S.J. All Authors read and approved the final article.

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