의학 박사 학위논문

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이 논문을 외과학 의학박사 학위논문으로 제출함

2016년 4월

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황대욱의 의학박사 학위논문을 인준함

2016년 7월

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Abstract

CX-4945, a CK2 inhibitor, Enhances Autophagy and Apoptosis in Pancreatic Cancer Cell Lines

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The Graduate School

Seoul National University

Background: Pancreatic cancer is the most lethal malignancy, with an overall 5-year survival rate of less than 5%. Although the need for more effective systemic therapeutics has long existed, there is only a marginal survival benefit from available adjuvant therapies. Because the inhibition of protein kinase CK2 has been reported as a novel therapeutic strategy for many cancers, we investigated the effects of CK2 inhibitors in pancreatic cancer cell lines.

Methods: The BxPC3, PATU8902 and Miapaca2 human pancreatic cancer cell lines were used in our experiments. CX-4945 was used as a novel CK2 inhibitor. Autophagy was analyzed by acridine orange staining, fluorescence microscope detection of punctuate patterns of GFP-tagged LC3 and immunoblotting for LC3. Cell survival, cell cycle and apoptosis analysis was performed.

Results: CX-4945 induced significant inhibition of proliferation and triggered autophagy in pancreatic cancer cell lines. This inhibition of proliferation was caused by direct inhibition of CK2α, which was required for autophagy and apoptosis in the pancreatic cancer cells. CX-4945 suppressed cell cycle progression in G2/M and induced apoptosis. The inhibition of CX-4945-induced autophagy was rescued by 3MA or siRNA against Atg7, which attenuated apoptosis in the pancreatic cancer cells.

Conclusion: CX-4945, a potent and selective inhibitor of CK2, effectively induces autophagy and apoptosis in pancreatic cancer cells, indicating that the induction of autophagy by CX-4945 may have an important role in the treatment of pancreatic cancer.

Keywords: Protein kinase CK2 inhibitor, CX-4945, Pancreatic cancer, Chemotherapy, Autophagy, Apoptosis

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1. Introduction

Pancreatic cancer is one of the most lethal and aggressive malignancies and is the fourth leading cause of cancer-related death with a dismal prognosis in western countries.[1] Despite progress in our knowledge of the molecular and genetic basis of this disease, the overall 5-year survival rate still does not exceed 5%, and only 20%-25% of all pancreatic cancer patients have the option of surgical resection.[1-3] Complete resection of pancreatic cancer with negative resection margins is the only chance for a cure, but only 15%-20% of patients who undergo curative resection are actually cured of their disease long term.[4,5] Even when curative resection is performed, the median survival after an R0 resection is 22 months, and more than half of patients develop systemic recurrence.[6,7] Therefore, many investigators have tried to find more effective systemic therapeutic strategies. However, the chemotherapy and radiation therapy presently available provide only marginal survival benefits, and there has been no clearly demonstrated chemotherapeutic agent for pancreatic cancer until now.[3]

Currently, marketed biologics and small molecules targeting kinases have been proven to be effective in treating well-characterized cancers. Protein kinase CK2, one such targeted kinase, is a constitutively active, highly conserved, ubiquitous serine/threonine kinase that is involved in a variety of cell signaling events related to the cell cycle, proliferation, and apoptosis.[8,9] CK2 overexpression and activity have been reported in many types of

cancers.[10] The overexpression of CK2 attenuates apoptosis of cancer cells, while inhibition of CK2 enhances cell death caused by drugs or radiation, suggesting its important regulatory role in the determination of cancer-cell fate.[11-13] As a results, CK2 is thought to be a significant target in cancer therapy.

CX-4945 is a small molecule inhibitor of the protein kinase CK2 and is a potent, selective orally bioavailable agent.[12] In this study, we investigated the effects of CX-4945 and the possibility of using CX-4945 as a novel therapeutic option for pancreatic cancer.

2. Materials and Methods

Cell culture and reagents

The human pancreatic cancer cell lines, BxPC-3, PATU8902, and MiaPaca-2 were obtained from the American Type Culture Collection (ATCC: Rockville, MD, USA) and the cells were cultured in RPMI- 1640 and DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37 ℃ in a 5% CO2 atmosphere. Gemcitabine was purchased from Sigma (St Louis, MO, USA) and Erlotinib, and CX-4945 was purchased from Selleck Chemicals Co.Ltd (Houston, TX, USA).

Cell survival assays

To perform MTT assays, cells were plated in 96-well sterile plastic plates. The cells were exposed to varying doses of CX-4945. After 72 h, 15 L of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 h. Crystalline formazan was solubilized with 100 μL of a 10% (w/v) SDS solution for 24 h. Absorbance at 595 nm was read spectrophotometrically using a microplate reader. Cell viability was determined using an ADAM-MC automatic cell counter (NanoEnTek, Seoul, Korea) according to the manufacturer's instructions.

Western blot analysis

Whole cell lysates were prepared using EBC lysis buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, and 5 U/mL aprotinin) and then centrifuged. The resulting supernatant (20 μg) was separated on an 8% - 12% SDS-PAGE gel and transferred onto PVDF membranes (Invitrogen). The membranes were blocked using 5% skim milk-PBS-0.1% Tween 20 for one hour at room temperature before being incubated overnight with a primary antibody specific for p-CK2α which was purchased from Sigma (St Louis, MO, USA), and a primary antibody specific for CK2α, which was purchased from Abcam (Cambridge, UK). Antibodies against mTOR, Caspase-3, Akt, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against P-mTOR (S2448), P-Akt (Ser473), cleaved PARP (Asp214), Atg7 and LC3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase–conjugated antibodies were used as secondary antibodies. The membranes were developed using ECL kits (PerkinElmer, Waltham, MA, USA).

Acridine orange staining

Autophagy was analyzed by staining cells with the vital dye, acridine orange (Sigma, St Louis, MO). The cells were trypsinized and incubated with acridine orange at a final concentration of 5 μg/mL for 30 min at 37 °C in a 5% CO2 atmosphere. Analyses were performed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using CellQuest software (Becton Dickinson). The results are representative of at least three, independent experiments, and the error bars signify standard deviations (SDs).

LC3-GFP expression

Cells were transfected with the pEGFP-LC3 plasmid (Addgene Inc., Cambridge, MA, USA) and cultured for 24 h. The cells were then treated with CX-4945 for 24 h. The punctate patterns produced by LC3 in the transfected cells were examined by fluorescence microscopy.

Cell cycle analysis

Cells were trypsinized, fixed in 70% ethanol at -20 ℃ from 60 minutes to a few days, incubated with 5 μL RNase (10mg/mL) and finally stained with 10 μL propidium iodide (1 mg/mL). Cellular DNA content in the treated cells was analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Apoptosis assay

Apoptosis was quantified using an Annexin V-FITC apoptosis kit (BD Biosciences, San Diego, CA, USA) in accordance with the manufacturer's instructions. In brief, cells were trypsinized, pelleted by centrifugation, and resuspended in Annexin V binding buffer (150 mM NaCl, 18 mM CaCl2, 10 nM HEPES, 5 mM KCl, and 1 mM MgCl2). FITC-conjugated Annexin V (1 μg/ml) and propidium iodide (50 μg/ml) were added to the cells which were incubated for 30 min at room temperature in the dark. Analyses were conducted on a FACScan (Becton Dickinson). The data were analyzed using CellQuest software (Becton Dickinson). The results are representative of at least three, independent experiments, and the error bars signify standard deviations (SDs).

Small interfering RNA transfection

Small interfering RNA (siRNA) oligonucleotides specific for Atg7, CK2 and an siRNA control were purchased from Santa Cruz Biotechnology (Santa Cruz). Cells were seeded into a 60-mm dish which was then left for 24 h. A 2 μL aliquot of siRNA solution (10 μM) and 5 μL of Lipofectamine 2000 (Invitrogen) were each mixed with 100 μL of serum-free RPMI-1640 medium. They were incubated for 20 min at room temperature after being

combined, and this mixture was then added to the cells that had been seeded on the dish. After 24 h, harvested cells were subjected to Western blot analysis. The cells were also processed for cell viability and apoptosis analysis.

3. Results

CX-4945 induced growth inhibition and autophagy in pancreatic cancer cell lines

We examined the effects of erlotinib, gemcitabine and CX-4945 on the pancreatic cancer cell lines BxPC-3, PATU8902 and MiaPaca-2. The cells were treated with increasing concentrations of drugs for 72 h, and growth inhibition was determined by MTT assay. Erlotinib and gemcitabine treatments did not show a significant growth inhibition in pancreatic cancer cells, while CX-4945 treatment showed growth inhibition in a dose-dependent manner (Figure 1A and 1B). Interestingly, we found that CX-4945 treatment triggered the accumulation of autophagic vacuoles (AV) (Figure 2A). To further confirm the induction of autophagy by CX-4945, we analyzed the expression of the autophagy marker, LC3-I/II, and the number of cells stained with acridine orange by performing Western blots and FACS analysis. As shown in Figure 2B and 2C, CX-4945 treatment led to the induction of endogenous LC3-II and acridine orange-positive cells. Furthermore, we found punctuate patterns of LC3 fluorescence signals in CX-4945-treated cells (Figure 2D). These results indicate that CX-4945 has the capability to trigger the induction of autophagy in pancreatic cancer cells.

CX-4945 induced cell cycle arrest and apoptosis in pancreatic cancer cells

Previous studies have shown that CX-4945 led to growth inhibition through the induction of the G2/M phase in various human cancer cells.[10,14-16] Thus, we investigated whether CX-4945 treatment could affect cell cycle distribution and cell death in pancreatic cancer cells. As shown in Figure 3A, when cells were treated with 10 μM of CX-4945 for 48 h, the number of cells in S/G2-M phase increased in all cell lines. In addition, we observed significant apoptotic cell death when cells were treated with CX-4945 for 72 h (Figure 3B). Consistent with these results, PARP and caspase-3 cleavage was also observed (Figure 3C). These results suggest that the CX-4945-induced antitumor effect in pancreatic cancer cells was associated with increased time spent in the S/G2-M phase of the cell cycle and apoptosis.

Inhibition of CK2α was required for autophagy and apoptosis of pancreatic cancer cells

Although CX-4945 is a potent and selective inhibitor of CK2, it is also a known inhibitor of other kinases, such as FLT3, PIM1, and CDK1. Thus, we examined whether CX-4945-induced autophagy and apoptosis is dependent on CK2α. Cells were transfected with CK2α siRNA. As shown in Figure 4A and 4B, the suppression of CK2α led to increased accumulation of autophagic vacuoles and the induction of endogenous LC3-II. In addition, we

found that the silencing of CK2α induced growth inhibition and apoptosis (Figure 4C and 4D). These results demonstrate that the antitumor effects of CX-4945 may be dependent on CK-2α.

Inhibition of CX-4945-induced autophagy attenuates apoptosis in pancreatic cancer cells

Although many studies have reported that pancreatic cancers require autophagy for tumor growth, the role of autophagy during cancer treatment is controversial.17-19 To further investigate whether the induction of autophagy is associated with sensitivity to CX-4945, we evaluated apoptotic cell death in the presence or absence of an autophagic inhibitor (3-methyladenine, 3MA) and Atg7-siRNA treatment. The number of autophagosome was significantly lower in cells pre-treated with 3MA (data not shown). The inhibition of autophagy by 3MA decreased cleaved PARP and caspase-3 levels and consequently led to a reduction in apoptotic cell death (Figure 5A). Consistent with the results from the 3MA treatment, the suppression of Atg7 reduced CX-4945-induced apoptosis (Figure 5B). Taken together, these results indicate that the induction of autophagy by CX-4945 may have an important role in the treatment of pancreatic cancer.

4. Discussion

Many targeting agents are on the path to becoming successful cancer therapeutics. However, there is no specific agent for pancreatic cancer, and most drugs and other therapeutic strategies have revealed limited impact on disease course and prognosis in pancreatic cancer. The combination of a lack of early diagnosis, highly complex tumor biology, and genetic heterogeneity interferes with effective systemic therapy. Curative resection is still the only chance for a cure in pancreatic cancer, and there is a marginal increase in survival using perioperative therapeutic strategies. Most clinical trials on 5-FU, gemcitabine, FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin), gemcitabine plus capecitabine, gemcitabine plus nab-paclitaxel, and other drugs, have revealed low effectiveness.[20-23] Among these, only a few trials have recently reported modest improvements in survival using combination treatments with gemcitabine and capecitabine, erlotinib, and FOLFIRINOX.[20-22] Although many trials targeting EGFR, VEGFR, RAS, MEK-ERK, and others are ongoing pancreatic cancer requires a novel therapeutic approach, and there are still unmet clinical needs for systemic therapy.

Kinase inhibitors are a novel therapeutic concept for pancreatic cancer.[24] Until now, several clinical trials have shown that new therapeutic strategies using kinase inhibition failed to improve survival in pancreatic cancer patients because pancreatic cancer differs from most other cancers in its lack

of a response to conventional anticancer treatment and its resistance to the induction of apoptosis.[25] Protein kinase CK2 is overexpressed in many types of cancer and cancer cells become reliant on the enzyme to sustain the necessary survival signaling. Inhibition of CK2 is an attractive, yet under-exploited approach for targeting processes essential for maintaining the cancer phenotype.[12] Therefore, the CK2 is another targeted promoter of the hallmarks of cancer and CX-4945 is a novel inhibitor of protein kinase CK2 that induces apoptosis in some cancers. In this study, gemcitabine and erlotinib had marginal effects on pancreatic cancer cell lines, but CX-4945 showed a more potent anti-tumor effect.

We observed that autophagy was induced by CX-4945 in pancreatic cancer cells. Several studies have reported that autophagy acts as a tumor promoter and protects tumor cells from cytotoxic drugs and stress.[17,26,27] These studies have indicated that autophagy helps tumor cells circumvent the stress induced by chemotherapy or environmental factors such as hypoxia or nutrient deficiency, and thus avoid apoptosis.28,29 Furthermore, the protective and anti-apoptotic role of autophagy has been reported, and some studies have shown that autophagy contributes to pancreatic cancer cell growth.[26,30-32] Autophagy is likely a survival response to unfavorable conditions; as such, it may play a negative role in cancer therapy outcomes. Indeed, autophagy allows for controlled catabolism of cellular macromolecules and therefore generates new metabolic substrates that

contribute to the maintenance of bioenergetic and biosynthetic homeostasis.[30] However, the actual role of autophagy in tumors is controversial, and some investigators have reported that cancer cells respond to drugs by inducing autophagy, resulting in cell death, especially when the normal apoptotic pathway is nonfunctional.[33,34] Our previous studies on CX-4945 showed that CX-4945 induced autophagy and inhibition of CK2 induced autophagic cell death.35 Thus, the correlation between autophagy and apoptosis induced by CX-4945 was investigated. This study demonstrated that autophagy induced by CX-4945 enhanced apoptosis in pancreatic cancer cell lines. Although several signaling pathways may be involved in autophagy, the mTOR-AKT signaling pathway is the most well-known. CX-4945 effectively downregulated the mTOR-AKT signaling pathway the examined pancreatic cancer cell lines, leading to autophagy. When autophagy is activated as a survival mechanism in cancer cells against the cytotoxic effects produced by drugs, it acts to protect of tumor cells. However, CX-4945 induces autophagy by itself and is thought to result in apoptosis of tumor cells. Therefore, the effect of CX-4945 should be interpreted differently from the correlations between conventional chemotherapeutic agents and autophagy. Accordingly, our results showing that siRNAs against Atg7 inhibit autophagy by attenuating apoptosis, further support this idea.

It is well known that CX-4945 is a specific inhibitor of the protein kinase CK2, but it was not clear whether the effects of CX-4945 were related to

CK2. We treated pancreatic cancer cell lines with CK2α-siRNA and found that apoptosis was induced by autophagy in these cells. This result confirmed that CX-4945 directly inhibits CK2, and CK2 should be considered an important target for pancreatic cancer treatment.

CX-4945 is the first orally bioavailable small molecule inhibitor of CK2, which makes it part of an entirely new class of targeted treatment for cancer, and it may be useful for the treatment of pancreatic cancer. Although, safety issues for this novel drug still exist, the clinical application of CX-4945 for pancreatic cancer should be considered because CX-4945 can modulate PI3K/Akt signaling and induce cell cycle arrest and apoptosis in pancreatic cancer cells. Clearly, further investigation into the anti-tumor effect of autophagy in pancreatic cancer cells will be required.

In conclusion, CX-4945, a potent and selective inhibitor of the protein kinase CK2, effectively induces autophagy and apoptosis in pancreatic cancer cells, suggesting its promising role in the treatment of pancreatic cancer.

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6. 요약(국문초록)

배경 및 목적: 췌장암은 근치적 절제를 시행한 이후에도 재발률이 매우 높고 생존률이 극히 불량한 것으로 잘 알려져 있다. 이에 췌장암을 전신 질환으로 다루어 치료할 필요성이 오래전부터 논의되고 있지만, 적용가능한 수술후 보조요법의 효과는 제한적인 상태이다. 이러한 새로운 항암제에 대한 필요성으로 다양한 연구가 진행되고 있는바, 이 연구에서는 CX-4945로 알려진 Protein kinase CK2 억제제의 췌장암에 대한 치료 효과를 알아보고, 그 기전을 확인하고자 하였다.

방법: 췌장암 세포주인 BxPC-3, PATU8902, MiaPaca-2를 이용하여 CK2 억제제인 CX-4945의 췌장암 세포에 대한 직접적인 사멸 효과를 in vitro에서 평가하였다. 이어서 이 사멸효과가 기존에 보고되어 있는 CX-4945의 자가포식현상과 관련이 있는지를 확인하기 위해, 자가포식현상의 표지자를 이용하여 이를 확인하였다.

결과: CX-4945단독요법으로 췌장암세포에 대하여 유의한 증식억제 및 자가포식현상의 유도가 관찰되었다. 췌장암에 대한 항암효과가 세포사멸에 의한 것인지, 또한 세포주기 중 어느 단계에서 발생하는 지를 확인하고자 하였고, CX-4945의 항암효과는 세포사멸의 표지자인 cleaved PARP 및 cleaved caspase-3로 확인되었으며, 세포주기는 G2/M에서 정지됨을 관찰하였다. 이러한 세포사멸과 자가포식현상이 CK2 억제로 인해 발생하는 지를 확인하기 위하여 CK2 siRNA로 CK2를 억제하였을 때,

세포사멸 및 자가포식현상이 모두 유의하게 감소함을 확인할 수 있었다. 이후 세포사멸과 자가포식현상 간의 관련성을 파악하기 위하여 자가포식현상을 억제하는 것으로 알려진 Atg-7 및 3MA 처리시 자가포식현상의 감소와 함께 세포사멸 역시 억제되는 것을 관찰할 수 있었다.

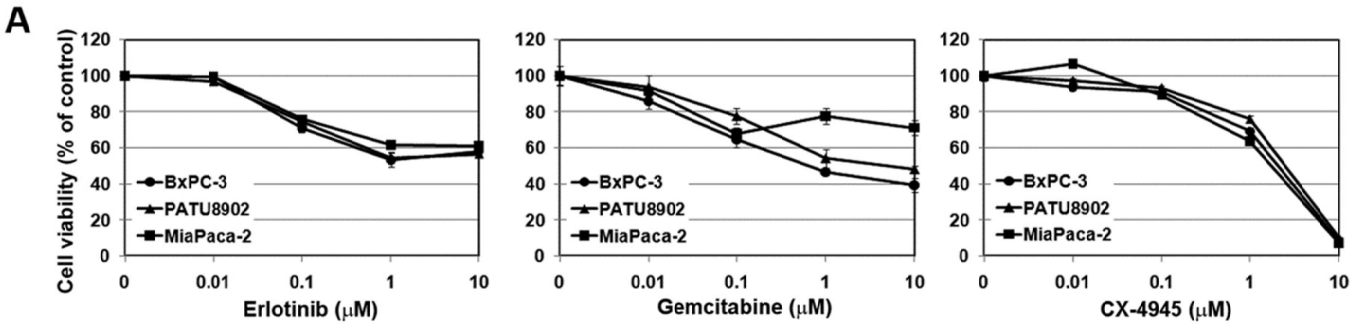
결론: 본 연구에서 CX-4945는 자가포식현상 및 세포사멸을 유도하여 췌장암세포에 의미있는 항암효과가 확인되었다. 따라서, 향후 췌장암에 대한 새로운 항암제로의 역할을 할 수 있을 것으로 예상된다.

주요어: Protein kinase CK2, 췌장암, 항암화학요법, CX-4945, 자가포식현상, 세포사멸

학번: 2008-30549

Figure 1. CX-4945 induced proliferative inhibition in pancreatic cancer cell lines

A, Cells were treated with Erlotinib, Gemcitabine, and CX-4945 for 72 h in a dose-dependent manner, and the rate of inhibition was determined by MTT assay. B, Cells were treated with different concentrations of CX-4945 for 72 h. Cell numbers were determined using an ADAM-MC, automatic cell counter. *$p<0.01$ and **$p<0.001$ compared with the control.


A
Cell viability (% of control)
120
100
80
60
40
20
0
BxPC-3
PATU8902
MiaPaca-2
0
0.01
0.1
1
10
Erlotinib (μM)
Gemcitabine (μM)
CX-4945 (μM)

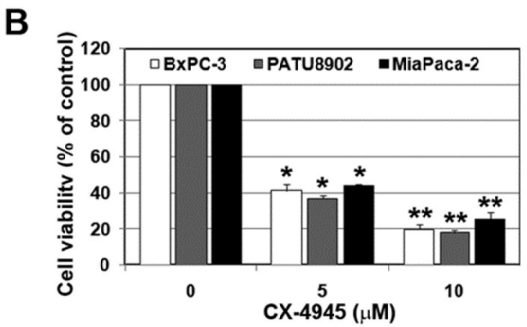

B
Cell viability (% of control)
BxPC-3
PATU8902
MiaPaca-2
*
**
0
5
10
CX-4945 (μM)

Figure 2. CX-4945 induced autophagy in pancreatic cancer cell lines

Cells were treated with or without CX-4945 (10 μM) for 48 h. A, Pictures showing autophagic vacuole (AV) formation were taken at 20x magnification. B, Cell lysates were subjected to Western blot analysis. C, Quantitative detection of acidic vesicular organelles by acridine orange staining of cells was determined by FACS analysis. *$p<0.01$ and **$p<0.001$ compared with the control. D, Cells were transfected with a plasmid to express LC3-GFP. After a 24 h transfection, the cells were treated with CX-4945 (10 μM) for 24 h. A punctate pattern of LC3 localization was visualized using immunofluorescence microscopy.

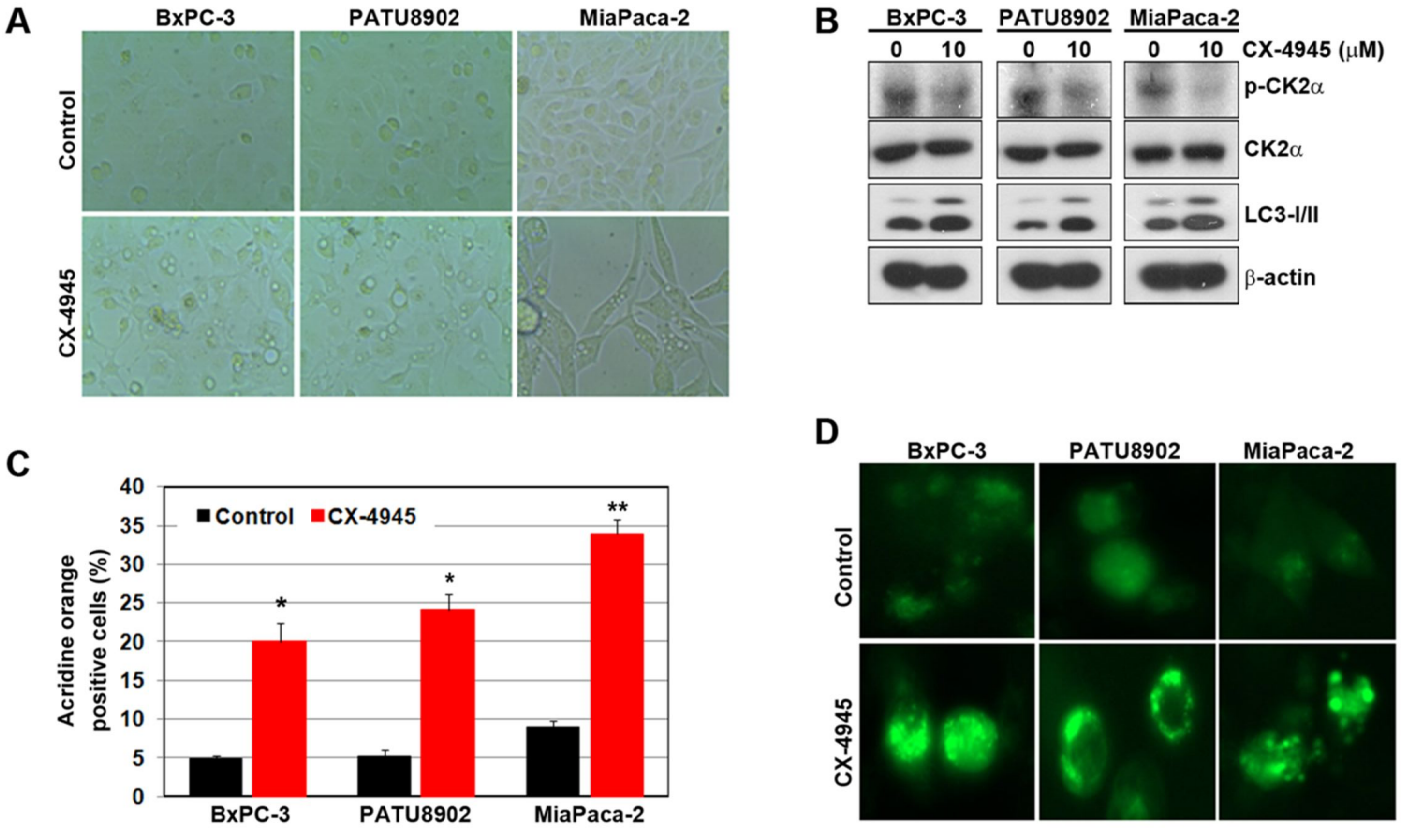

A
BxPC-3
PATU8902
MiaPaca-2
Control
CX-4945
B
BxPC-3
PATU8902
MiaPaca-2
0
10
0
10
0
10
CX-4945 (μM)
p-CK2α
CK2α
LC3-I/II
β-actin
C
Acridine orange positive cells (%)
Control
CX-4945
40
35
30
25
20
15
10
5
0
*
*
**
BxPC-3
PATU8902
MiaPaca-2
D
BxPC-3
PATU8902
MiaPaca-2
Control
CX-4945

Figure 3. CX-4945 induced G2/M cell cycle arrest and apoptosis in pancreatic cancer cell lines

Cells were treated with CX-4945 (10 μM) for 48 h or 72 h. A, After 48 h, the cells were harvested, treated with RNase, and stained with propidium iodide (PI). Cell cycle distribution was analyzed by flow cytometry. B and C, After 72 h, the cells were harvested and apoptosis was assessed by Annexin V-FITC/PI staining and flow cytometry. *$p<0.01$ and **$p<0.001$ compared with the control. Equal amounts of whole cell lysates (20 μg) were subjected to electrophoresis and the proteins were analyzed by Western blotting using the indicated antibodies.

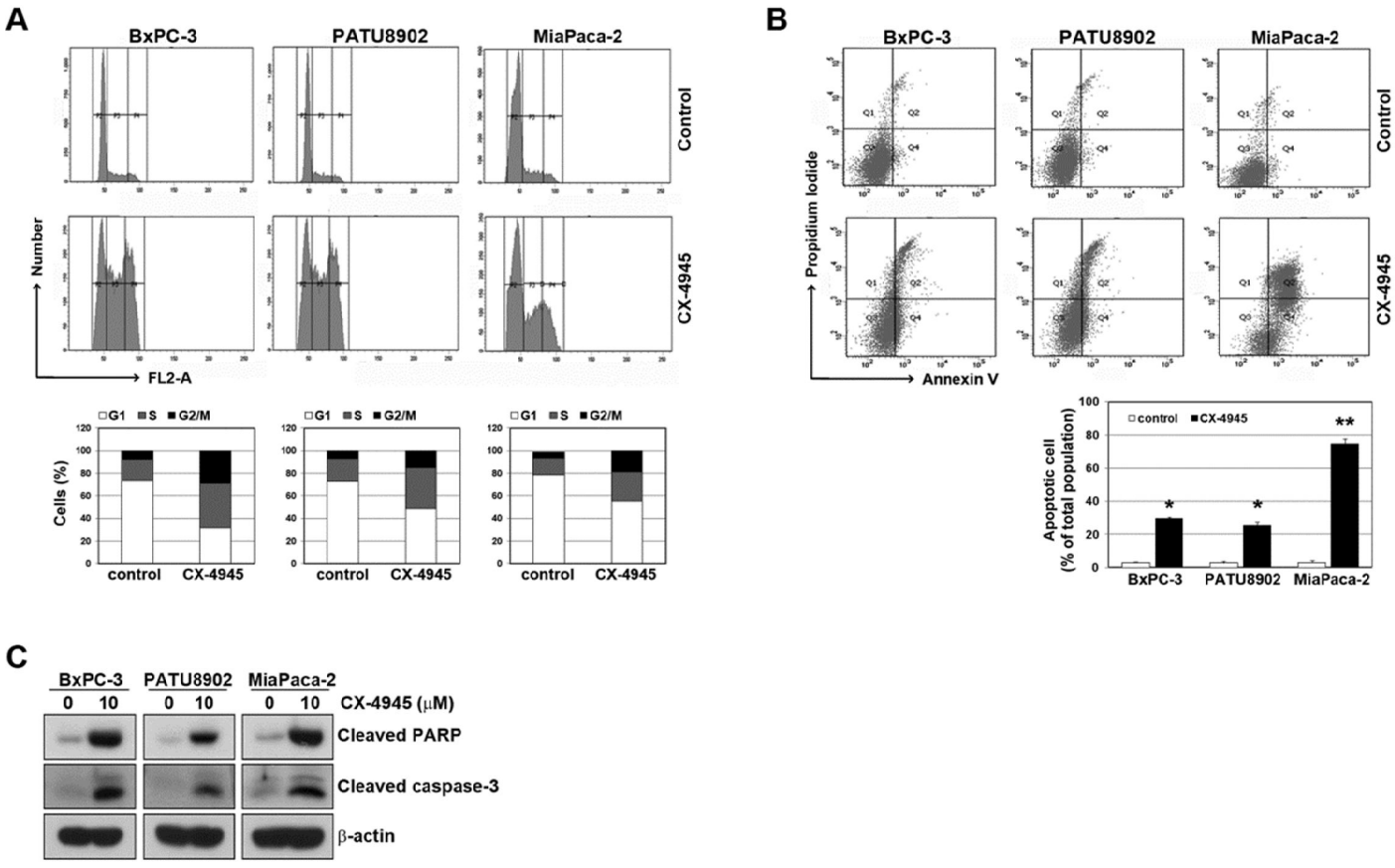

A
BxPC-3
PATU8902
MiaPaca-2
Control
CX-4945
Number
FL2-A
G1 S G2/M
Cells (%)
control
CX-4945
B
BxPC-3
PATU8902
MiaPaca-2
Control
CX-4945
Propidium Iodide
Annexin V
Q1
Q2
Q3
Q4
control
CX-4945
Apoptotic cell
(% of total population)
*
*
**
BxPC-3
PATU8902
MiaPaca-2
C
BxPC-3
PATU8902
MiaPaca-2
0 10
0 10
0 10
CX-4945 (μM)
Cleaved PARP
Cleaved caspase-3
β-actin

Figure 4. Down-regulation of CK2α by siRNA treatment induced autophagy and apoptosis in pancreatic cancer cell lines

Cells were transfected with control or CK2α-siRNA (100 nM) for 48 h. A, The suppression of CK2α and the induction of LC3-II was analyzed by Western blot. B, Pictures showing autophagic vacuole (AV) formation were taken at 20x magnification. C, Cell numbers were determined with an ADAM-MC automatic cell counter. **$p<0.001$ compared with the control. D, The induction of apoptosis was analyzed by Western blotting.

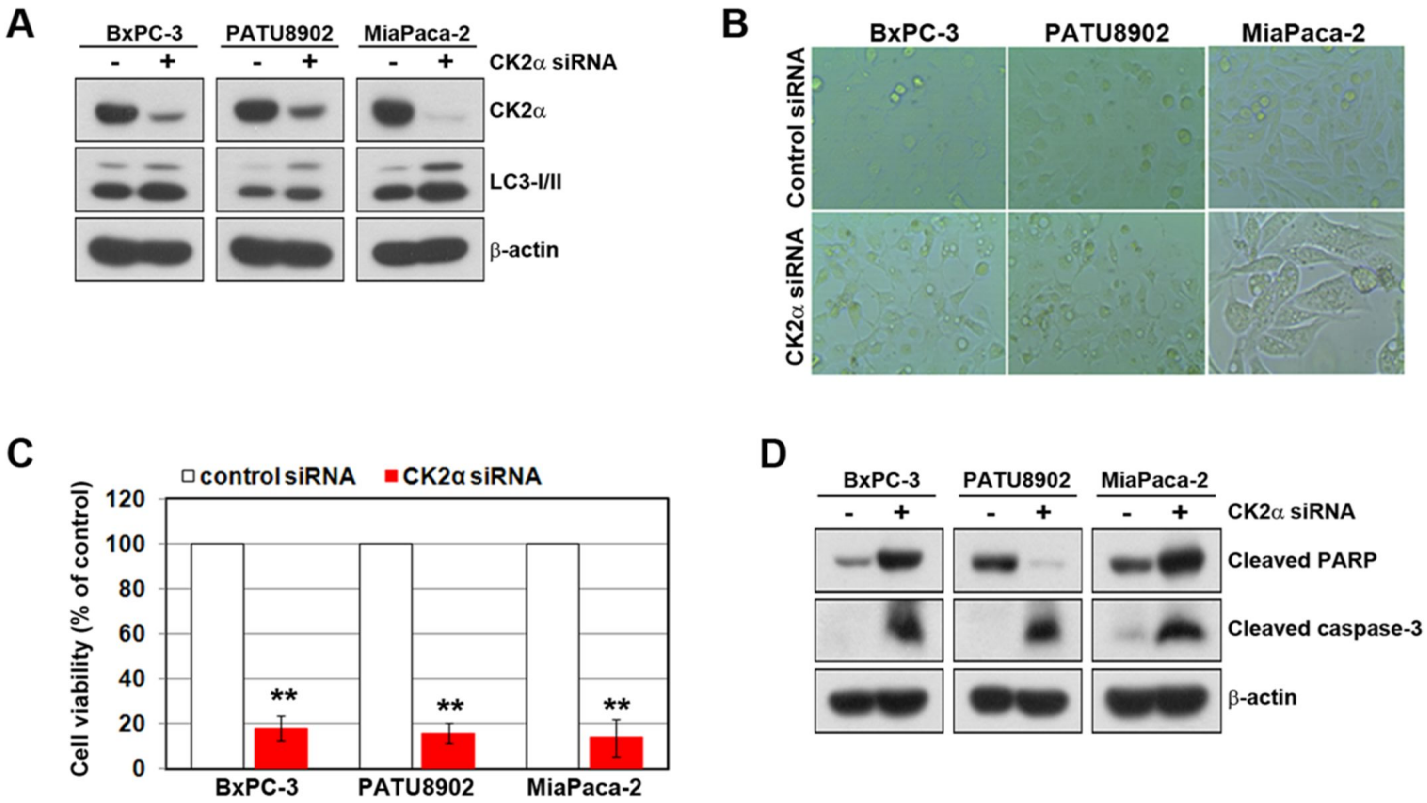
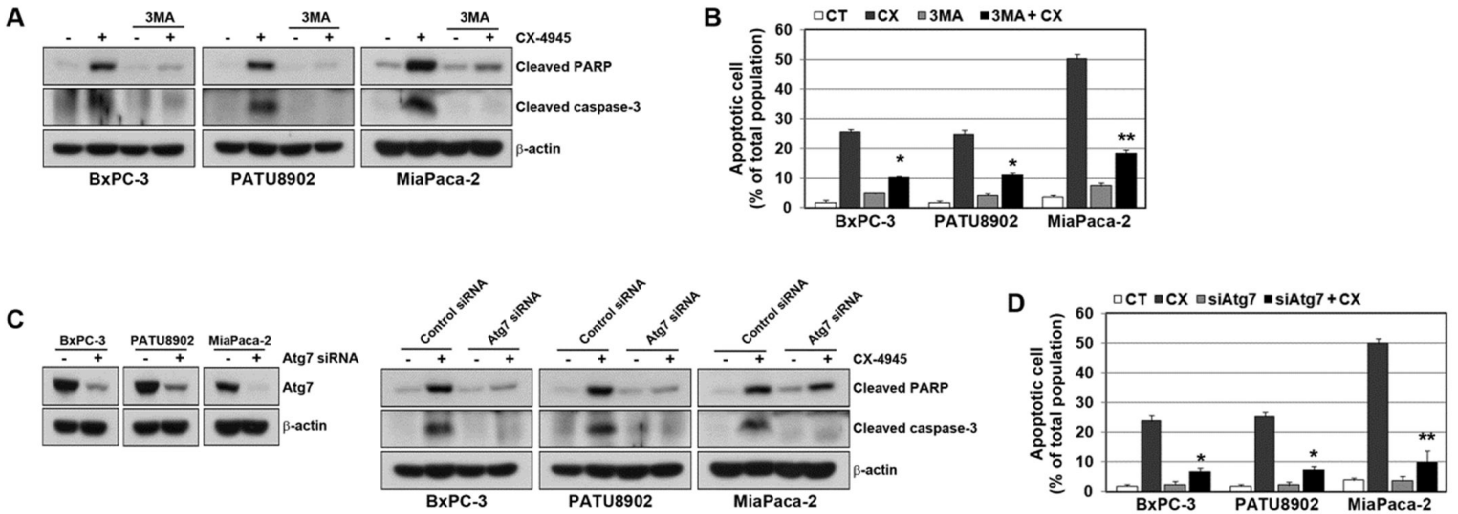

A
BxPC-3
PATU8902
MiaPaca-2
- + - + - +
CK2α siRNA
CK2α
LC3-I/II
β-actin
B
BxPC-3
PATU8902
MiaPaca-2
Control siRNA
CK2α siRNA
C
control siRNA
CK2α siRNA
Cell viability (% of control)
120
100
80
60
40
20
0
**
**
**
BxPC-3
PATU8902
MiaPaca-2
D
BxPC-3
PATU8902
MiaPaca-2
- + - + - +
CK2α siRNA
Cleaved PARP
Cleaved caspase-3
β-actin

Figure 5. Inhibition of CX-4945-induced autophagy led to decreased apoptosis

A and B, Cells were treated with CX-4945 for 72 h in the presence or absence of 3MA (1mM). C and D, The cells were transfected with Atg7- siRNA instead of 3MA. Cleavage of PARP-1 and caspase-3 was shown by Western blot analysis. B, Apoptosis was assessed by Annexin V-FITC/PI staining and flow cytometry. The results are representative of at least 3 independent experiments, and the error bars signify standard deviations (±SDs). *$p<0.01$ and **$p<0.001$ compared with CX-4945 treatment.


A
3MA
CX-4945
Cleaved PARP
Cleaved caspase-3
β-actin
BxPC-3
PATU8902
MiaPaca-2
B
CT
CX
3MA
3MA + CX
Apoptotic cell (% of total population)
C
Atg7 siRNA
Atg7
Control siRNA
D
siAtg7
siAtg7 + CX