A novel and promising therapeutic approach for treating pancreatic cancer: Nectin-4-targeted antibody-drug conjugates alone or combined with autophagy inhibitors

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Abstract. Antibody-drug conjugates (ADCs) are rapidly advancing the treatment of solid tumors, and Nectin-4-targeted ADCs have been approved by the FDA to treat certain cancers. Although Nectin-4 is overexpressed in the tissues of patients with pancreatic cancer, whether Nectin-4-targeted ADCs can effectively treat pancreatic cancer remains unclear. The present study evaluated the therapeutic effects and mechanisms of Nectin-4-targeted ADCs in pancreatic cancer. A Nectin-4-directed ADC was chosen, Nectin-4-MMAE, which triggered apoptosis and induced cell death in the Nectin-4-positive pancreatic cancer cell lines BxPC-3 and YAPC. Nectin-4-MMAE also induced autophagy in BxPC-3 and YAPC cells by inactivating the AKT/mTOR pathway. The entire autophagy process was observed by electron microscopy and laser confocal microscopy. The autophagy inhibitors LY294002 and chloroquine significantly increased the lethal effects of Nectin-4-MMAE on BxPC-3 and YAPC cells by inducing apoptosis. In the xenograft tumor model, Nectin-4-MMAE alone elicited potent antitumor effects. When Nectin-4-MMAE was combined with autophagy inhibitors, the tumor burden of mice was decreased compared with treatment with either drug alone. The present study confirmed the potent therapeutic effects of Nectin-4-MMAE against pancreatic cancer, and its unique antitumor mechanism provides new approaches to treatment.

Introduction

Pancreatic cancer is a highly lethal cancer, accounting for nearly 5% of cancer-related deaths worldwide (1). Numerous patients with pancreatic cancer are diagnosed in the late stages of the disease because there are no apparent symptoms, resulting in missed opportunities for surgical treatment. The main treatments for pancreatic cancer are pancreatectomy and drug chemotherapy, but the prognosis for patients is poor. Despite advances in combination chemotherapy, the median survival of patients with pancreatic cancer is only 10-12 months (2,3). Due to the limited efficacy of current treatment options, targeted therapies are receiving attention for addressing this unmet clinical need (4). Of significant note, antibody-drug conjugates (ADCs) have become a popular research topic in antitumor drug development, combining the advantages of precise targeting and efficient elimination to achieve exact and efficient elimination of cancer cells. Since the U.S. FDA first approved the ADC drug Mylotarg in 2000, 13 ADCs have reached the market. However, no ADCs have yet been authorized by the FDA for the treatment of pancreatic cancer (5). ADC treatment for pancreatic cancer has shown promise in preclinical studies. Presently, clinical trials of ADC drugs targeting pancreatic cancer are also underway, including ADCs targeting claudin18.2: SHR-A1904 (NCT04928625), TROP2: IMMU-132 (NCT01631552) and c-MET: RC108 (NCT05628857). Despite the advances in the efficacy of these ADCs in the treatment of pancreatic cancer, there are few studies investigating the specific mechanisms of ADCs in pancreatic cancer, and no studies of Nectin-4-targeted

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ADCs in pancreatic cancer have been reported to the best of the authors' knowledge.

Nectin-4, a type I transmembrane cell adhesion protein, is expressed at low levels in adults under physiological conditions and overexpressed in various tumor tissues, and therefore is an attractive antigen target for ADC (6). The first FDA-approved Nectin-4-targeted ADC, enfuzumab vedotin, is a treatment for urothelial cancer (7). According to the literature, the overall positivity rate of pancreatic cancer for Nectin-4 was reported to be ~71%, and the expression of Nectin-4 in pancreatic cancer tissue was markedly higher than that in normal pancreatic tissue (8). Another study found that Nectin-4 may be closely associated with Capan-2 and BxPC-3 cell proliferation. Nectin-4 may contribute to tumor cell proliferation in human pancreatic cancer, according to immunohistochemistry experiments (9). These findings suggest that Nectin-4 may be a potential target in pancreatic cancer and that Nectin-4-targeted ADCs may be a potential therapeutic agent to support further clinical studies and treatment. In the present study, experiments were performed using Nectin-4-MMAE, 9MW2821, which consists of an anti-Nectin-4 antibody (MW282 mAb), the IDconnect linker and the cytotoxic molecule MMAE.

Autophagy is an important regulator of cancer cell metabolism, and its role in various solid tumors is dynamic and dependent on the environment (10,11). On the one hand, autophagy is a cytoprotective mechanism that degrades damaged, degenerated and senescent cells to provide raw materials for cell regeneration and repair (12). On the other hand, excessive autophagy leads to metabolic stress and cell death (13,14). Tumor cells induce autophagy under hypoxic and starvation conditions; inhibiting autophagy significantly increases tumor death and suppresses tumor cell proliferation (15). Wang *et al* (16) reported that a combination of Nectin-4-MMAE and autophagy inhibitors exhibited synergistic antitumor effects in bladder cancer. The mechanism of autophagy in Nectin-4-MMAE treatment of pancreatic cancer also merits further exploration.

The present study aimed to investigate the possible antitumor effects of Nectin-4-MMAE on pancreatic cancer both *in vivo* and *in vitro*. Moreover, the current study delved into the significant roles of autophagy and apoptosis in Nectin-4-MMAE-induced pancreatic cancer cell death. Autophagy inhibitors were combined with Nectin-4-MMAE to evaluate the possibility of enhancing its antitumor effects and to explore the feasibility of this combination therapy for treating pancreatic cancer.

Materials and methods

Reagents and antibodies. 9MW2821 was provided by Mabwell (Shanghai) Biotechnology Co., Ltd. LY294002 was purchased from Selleck Chemicals (cat. no. S1105). Antibodies from Cell Signaling Technology, Inc. used in the present study were as follows: anti-PARP antibody (cat. no. 9542), anti-cleaved caspase-9 antibody (cat. no. 9502), anti-cleaved caspase-3 antibody (cat. no. 9662), anti-phospho-AKT antibody (Ser473; cat. no. 4060), anti-phospho-mTOR antibody (Ser2448; cat. no. 2971), anti-phospho-p70s6k antibody (Ser371; cat. no. 9208), anti-phospho-4EBP1 antibody (Thr45; cat. no. 2855), anti-LC3 antibody (cat. no. 3868) and anti-SQSTM1 antibody (cat. no. 8025). All of the primary antibodies were derived from rabbits and the dilution ratio was 1:1,000. HRP-conjugated anti-rabbit IgG secondary antibody (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) and anti-β-actin antibody (rabbit; 1:5,000; cat. no. GB15003-100; Wuhan Servicebio Technology Co., Ltd.) were also used.

Cell culture. The human pancreatic cancer cell lines BxPC-3, YAPC and PANC-1 were purchased from the Shanghai Typical Cultures Depository of the Chinese Academy of Sciences. PANC-1 cells were cultivated in DMEM media (cat. no. MA 0212; Dalian Meilun Biology Technology Co., Ltd.), while BxPC-3 and YAPC cells were cultured in RPMI-1640 media (cat. no. MA 0215; Dalian Meilun Biology Technology Co., Ltd.). Cell lines were maintained in an environment with 5% carbon dioxide, suitable humidity, and a temperature of 37˚C. It was confirmed that all cell lines are mycoplasma-free through routine mycoplasma testing.

Cellular Nectin-4 expression assay. Cells were resuspended in ice-cold PBS. Then 100 μl (1x10^6 cells/ml) of the cell suspension and 2 μg/ml of anti-Nectin-4 antibody were added to a centrifuge tube. The suspension was incubated for 30 min at 4˚C while being protected from light. The cells were washed three times and fluorescent-conjugated secondary antibody (1:1,000; cat. no. 398004; BioLegend, Inc) was added to the appropriate tubes. The suspension was incubated at 4˚C in the dark for 30 min. After washing the cells three times, they were resuspended in PBS for subsequent analysis. Cells were analyzed by flow cytometry (CytoFLEX; Beckman Coulter, Inc.) using the software CytExpert 2.4 (Beckman Coulter, Inc.).

Cell viability assay. After the indicated co-incubation time, 100 μl of MTT solution (cat. no. MB4698; Dalian Meilun Biology Technology Co., Ltd.) was added to the cells and incubated at 37˚C for 4 h. The methylated product was dissolved with DMSO, and the plates were gently shaken at room temperature for 5 min. An enzyme marker was used to detect the absorbance at 490 nm.

Confocal microscopy. The internalization of ADCs and the subsequent autophagic flow were investigated using confocal fluorescence imaging. Using an Alexa Fluor® 488 Protein Labelling Kit (cat. no. A10235; Thermo Fisher Scientific, Inc.), the ADCs were labeled with Alexa Fluor® 488 and incubated for the specified amount of time. ADCs were then applied to YAPC and BxPC-3 cells for specified durations. Autophagosomes and lysosomes were identified using a Cyto-ID Autophagy Detection Kit (cat. no. ENZO-51031K200; Enzo Life Sciences, Inc.). The cells were observed using a Carl Zeiss LSM710 (Carl Zeiss AG) confocal microscope.

Western blot analysis. The cells were lysed using RIPA buffer (cat. no. P0013D; Beyotime Institute of Biotechnology). The total protein concentration was measured after the supernatant was collected using the BCA Protein Assay Kit (cat. no. P0012; Beyotime Biotechnology). Loading buffer was added and the proteins were heated for 8 min at 100˚C to denature the proteins. SDS-PAGE gels (8-15%) were

loaded with equal volumes of proteins (20 μg per lane) and then underwent electrophoretic separation. The proteins were subsequently transferred to a PVDF membrane. Next, the membrane was blocked with 5% BSA (cat. no. MB4219; Dalian Meilun Biology Technology Co., Ltd.) for 2 h at room temperature, and incubated overnight with the primary antibody at 4˚C, followed by the secondary antibody for 2 h at room temperature. Using an ECL kit (cat. no. MA0186; Dalian Meilun Biology Technology Co., Ltd.), the membrane protein bands were observed. Imaging was performed using a gel imager from Bio-Rad Laboratories, Inc. Densitometric analysis was carried out using Image LAB 5.2 software, and the grayscale values of the bands were calculated using ImageJ (National Institutes of Health).

Apoptosis analysis. Apoptosis was identified by using the Annexin V-FITC/PI Apoptosis Detection Kit (cat. no. MA0220; Dalian Meilun Biology Technology Co., Ltd.). Cells were processed and stained according to the manufacturer's protocol. After $1x10^5$ cells were centrifuged, the supernatant was discarded. 195 μl of Annexin V-FITC binding buffer was added and the cells were gently resuspended. Then, 5 μl of Annexin V-FITC and 10 μl of propidium iodide staining solution were added and gently mixed. Incubate the samples in the dark for 15 min before performing the detection on the instrument. Using a flow cytometer, cells were found and examined (CytoFLEX; Beckman Coulter, Inc.) using the software CytExpert 2.4 (Beckman Coulter, Inc.).

Transmission electron microscopy. The autophagic structure of BxPC-3 cells was determined using transmission electron microscopy. When the cell density was ~70%, the cells were digested with EDTA-free digestive enzymes. After centrifugation (300 x g, 3 min, 20˚C), the supernatant was discarded and the cells were fixed using 2.5% glutaraldehyde fixative. The samples were kept at 4˚C. The embedding, sectioning and subsequent image acquisition work were entrusted to Wuhan Servicebio Technology Co., Ltd. The samples were recorded using a transmission electron microscope at magnifications of x1,500 and x6,000.

Tumor xenograft model. BALB/c nude male mice (n=25, 18 g, 6-weeks old) were purchased from the Shanghai Model Organisms Center. Male mice based the stable hormone level making it easier to determine the antitumor efficacy of ADCs. All animals were kept in the Experimental Animal Center of Fudan University under a 12/12 h light/dark cycle (17). The temperature in the animal room was controlled between 20-26˚C. The mice were given full access to regular food and water during the study. BxPC3 cells suspended in PBS were subcutaneously injected at a density of $1x10^7$ cells per mouse to establish mouse xenograft tumor model. A total of 5 groups of mice were randomly selected, with 5 mice in each group. The treatments were as follows: Intraperitoneal injection of PBS; single dose intravenous injection of Nectin-4-MMAE (3 mg/kg); intraperitoneal injection of chloroquine (50 mg/kg; cat. no. C6628; MilliporeSigma) once a day; combination of Nectin-4-MMAE and CQ; and twice a week intravenous injection of the positive control drug gemcitabine (50 mg/kg). Tumor volume

in mice was calculated by measuring length and width (volume=0.5 x length x width^2). After 21 days of drug administration, the experimental animals were euthanized by cervical dislocation operation. The researchers observed the mice directly for undulating movements of the chest to determine respiratory arrest. At the same time, the left side of the mice's chest was gently touched with a finger to determine whether the heartbeat had stopped by touch. When the mice's vital signs completely stopped and there was no sign of recovery after continuous observation, the mice's death status was formally confirmed, and then the subsequent experimental process steps were carried out. Sections of excised tumor tissue were prepared for H&E staining.

Hematoxylin and eosin (H&E) staining. Sectioning and H&E staining were both entrusted to Wuhan Servicebio Technology Co., Ltd. The tumor tissue was collected and fixed in a 4% paraformaldehyde solution for 24 h. Subsequently, paraffin embedding was carried out, and the tissue was sectioned into slices with a thickness of 3-4 μm. The slices were then deparaffinized in xylene and hydrated through a series of alcohol solutions with decreasing concentrations. Next, the slices were stained with H&E. After staining, the slices were dehydrated with anhydrous ethanol and made transparent with xylene. Finally, the treated slices were mounted and observed under the Whole Slide Scanning System (Olympus VS200).

Statistical analysis. Adobe Photoshop and Illustrator were used to create the graphs. GraphPad Prism 8.3 (Dotmatics) and Excel (version 2019; Microsoft Corporation) were used to analyze the data, and the results of three independent replicate experiments are shown as the mean ± SD. Groups were compared using a one-way ANOVA followed by Dunnett's or Tukey's post hoc tests and the unpaired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ADCs targeting Nectin-4 induce apoptosis in pancreatic cancer cell lines. Flow cytometry screening was performed on two Nectin-4 positive pancreatic cancer cell lines, BxPC-3 and YAPC, and 1 Nectin-4 negative cell line, PANC-1, for subsequent experiments (Fig. 1A). YAPC and BxPC-3 cells were cultured with 10 μl (2 μg/ml) of Nectin-4-MMAE labeled with Alexa Fluor® 488. After 30-90 min of treatment, intracellular green fluorescence was observed using laser confocal microscopy, indicating that Alexa Fluor® 488-labeled Nectin-4-MMAE bound to the cell surface receptors and enter the cell for internalization (Fig. 1B and C). Using an MTT assay, it was found that the cytotoxicity of BxPC-3 and YAPC cells increased significantly as the concentration of Nectin-4-MMAE increased after 72 h, while PANC-1 cell viability was not significantly affected (Fig. 1D). The cytotoxicity of ADCs is not potent enough, which might depend on the targets, the cell lines and the properties of ADCs.

To explore the mechanism of cell elimination, cells were analyzed for apoptosis by staining with Annexin V/PI after BxPC-3 and YAPC cells were treated with different concentrations of Nectin-4-MMAE for 72 h. As the concentration
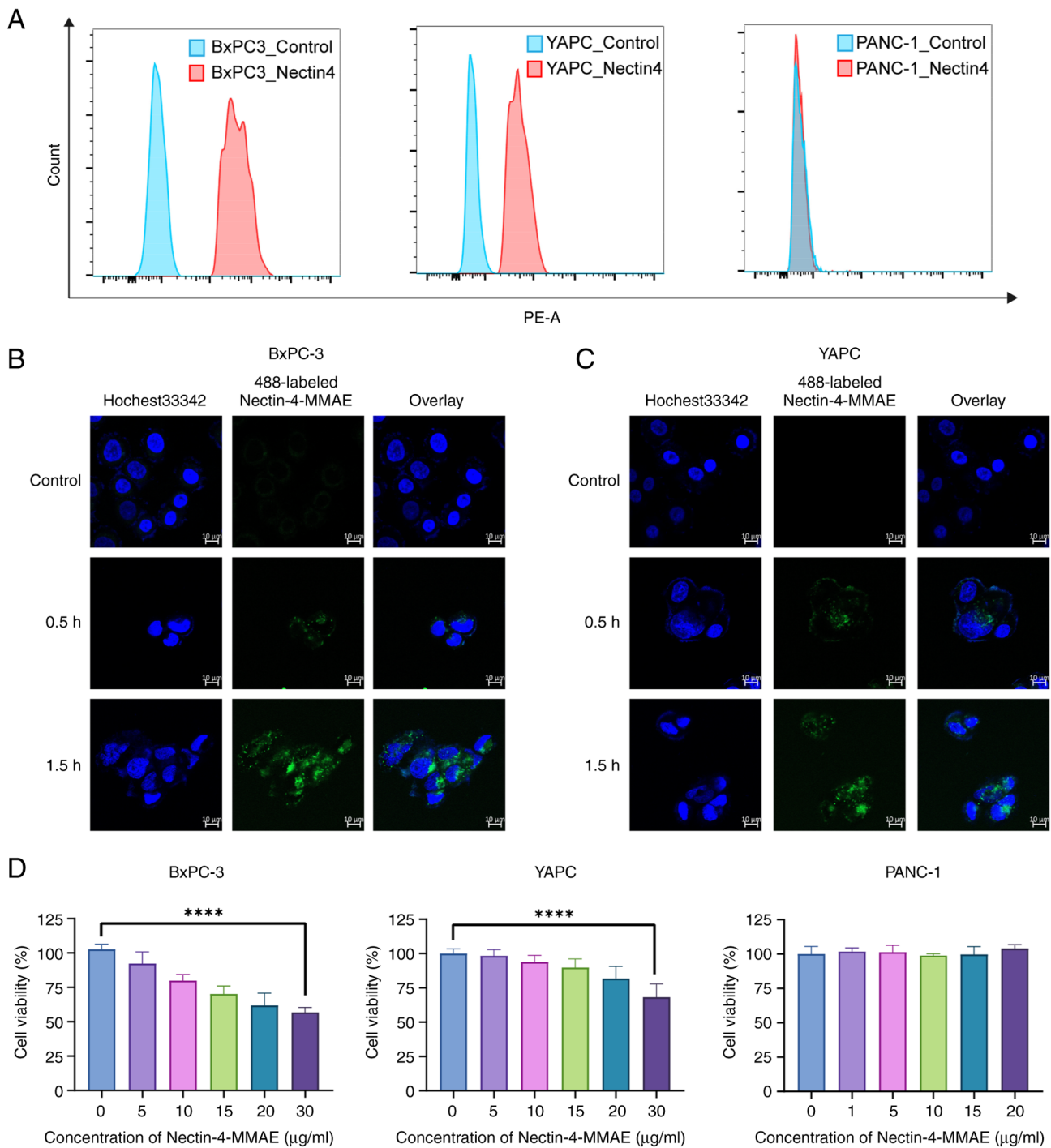
Figure 1. Nectin-4-MMAE's cytotoxicity and endocytosis. (A) Nectin-4 positive cell lines BxPC-3 and YAPC were selected by flow cytometry. (B) Internalization of Nectin-4-MMAE into BxPC-3 cells. (C) Internalization of nectin-4-MMAE into YAPC cells. Blue: Live cells stained with Hoechst 33342; green: Nectin-4-MMAE labeled with Alexa Fluor™ 488. (D) Cytotoxic effects of Nectin-4-MMAE on BxPC-3, YAPC and PANC-1 cells as assessed by MTT assays. ****$P<0.0001$.

of Nectin-4-MMAE within BxPC-3 and YAPC cells rises, the overall number of both early apoptotic and late apoptotic cells similarly increased (Fig. 2A-D). The expression of apoptosis-related proteins was analyzed in YAPC and BxPC-3 cells by western blotting (Fig. 3A and B). As the concentration of Nectin-4-MMAE increased, there was a significant rise in apoptosis-related protein levels. This finding suggested that Nectin-4-MMAE caused cell death by cleaving PARP downstream and through apoptosis by activating the caspase

cascade. The results suggested that Nectin-4-MMAE induced apoptosis and cytotoxicity in BxPC-3 and YAPC cells.

Nectin-4-directed ADC induces autophagy in BxPC-3 and YAPC cells. Autophagosomes in BxPC-3 cells after Nectin-4-MMAE treatment were observed using transmission electron microscopy (Fig. 4A). The autophagosomes had a double-membrane structure (marked by red arrows). This result suggested that Nectin-4-MMAE resulted in the
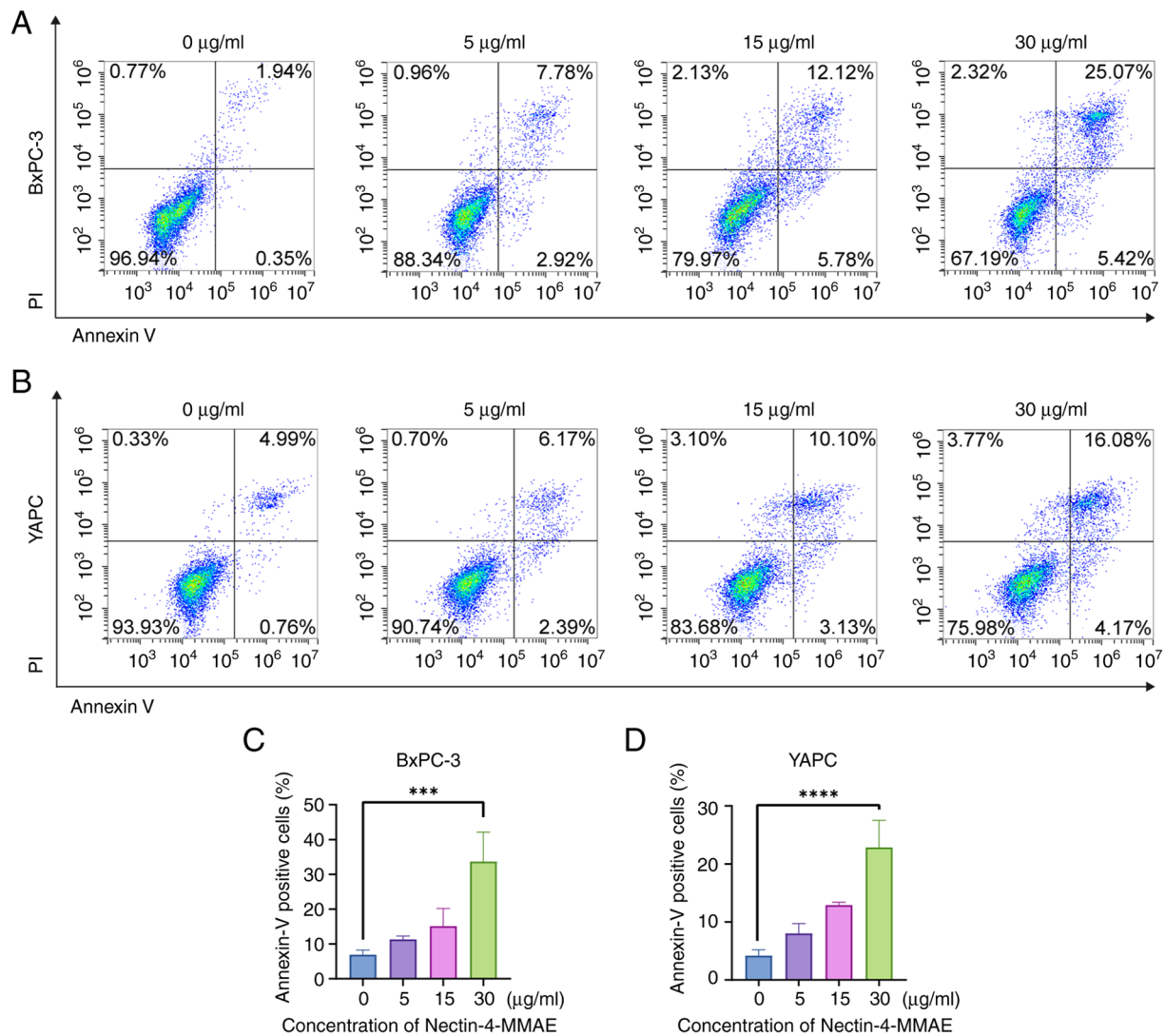
Figure 2. Nectin-4-MMAE induces apoptosis in BxPC-3 cells and YAPC cells. (A-D) Annexin V-FITC/PI staining for apoptosis of (A and C) BxPC-3 cells and (B and D) YAPC cells induced by nectin-4-MMAE. ***P<0.001 and ****P<0.0001.

production of intracellular autophagic vesicles. To further confirm the relationship between autophagy and ADCs, the cells were divided into three groups: The negative control group, the Nectin-4-MMAE group (15 µg/ml), and the positive control rapamycin group. BxPC-3 and YAPC cells were plated and incubated with the corresponding drugs for 12 h. The cells were stained with the autophagosome dye Cyto-ID and observed by confocal microscopy. It was found that the intensity of green fluorescence significantly increased in the Nectin-4-MMAE groups, which was consistent with the results of the positive control rapamycin group (Fig. 4B and C). In addition, the expression of the autophagy marker protein SQSTM1 decreased as the Nectin-4-MMAE concentration increased, and LC3 II expression increased as the Nectin-4-MMAE concentration increased, suggesting an increase in autophagy (Fig. 4D-F).

Nectin-4-directed ADCs induce autophagy flux in BxPC-3 and YAPC cells. BxPC-3 and YAPC cells were treated with 15 µg/ml Nectin-4-MMAE for different time intervals. Autophagosome formation was detected at 12 h and peaked at 24 h. Lysosome formation was detected in BxPC-3 and YAPC cells after 24 h. After 48 h, lysosomes were observed in BxPC-3 cells, while lysosomes disappeared in YAPC cells. Yellow fluorescence was observed in the merged image at 24 h and disappeared at 48 h, indicating that autophagosomes become autophagic lysosomes and were subsequently degraded. This result demonstrated the entire process of Nectin-4-MMAE-induced autophagic flux (Fig. 5A and B).

To improve understanding of how Nectin-4-MMAE induces autophagy, the phosphorylation levels of the upstream regulators of the classical Akt/mTOR autophagy pathway were further examined through western blotting. It was identified that as the quantity of Nectin-4-MMAE increased, phosphorylated (p)-Akt, p-mTOR, p-p70S6k and p-4EBP1 protein levels dramatically decreased (Fig. 5C-F). These results revealed that the Akt/mTOR signaling pathway was inactivated with increasing concentrations of Nectin-4-MMAE. Combining these results showed that autophagy was induced by inactivating the classical Akt/mTOR pathway, and Nectin-4-MMAE achieved the goal of eliminating tumor cells through autophagy and apoptosis.
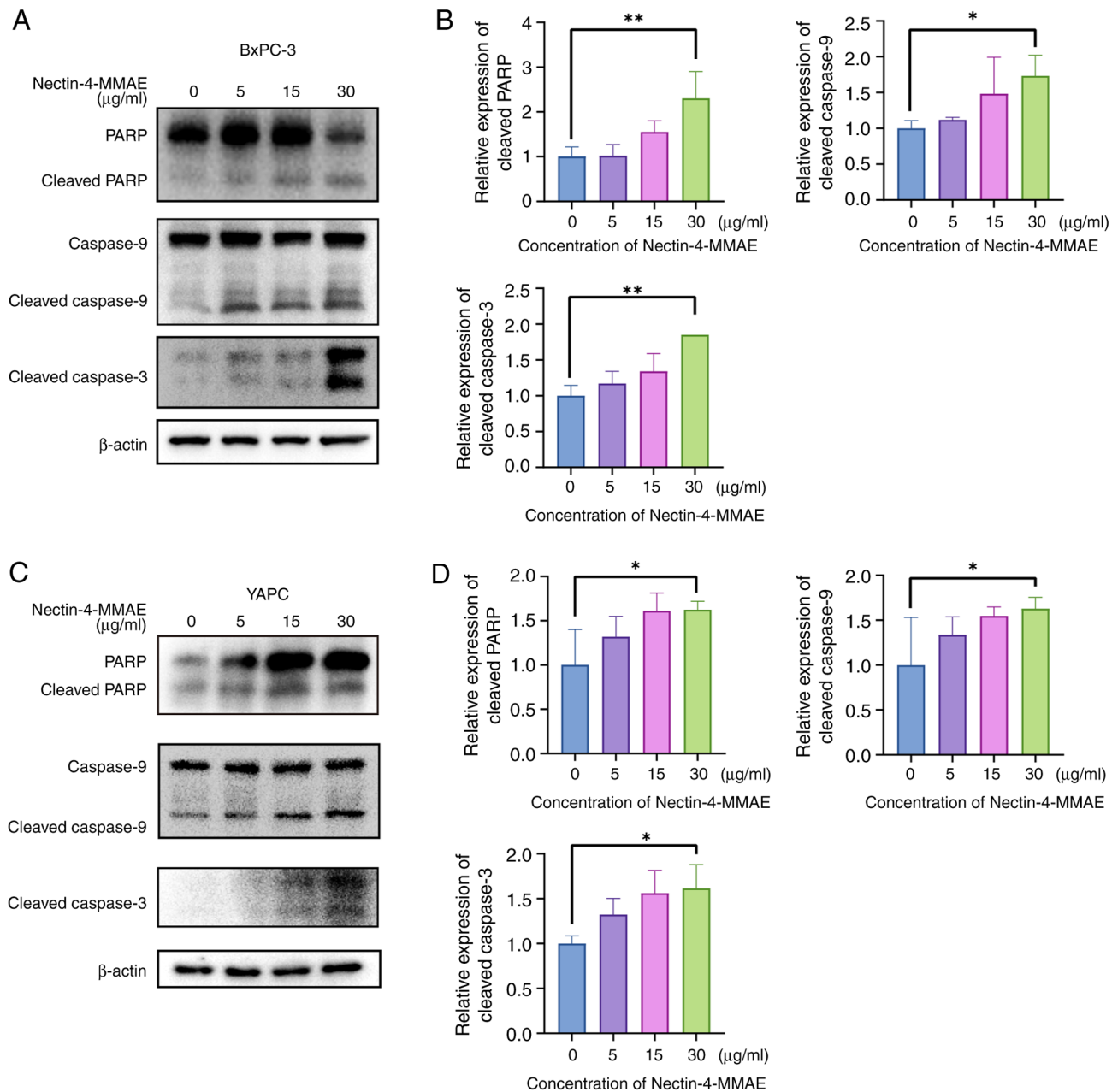
Figure 3. Nectin-4-MMAE induces apoptosis in BxPC-3 cells and YAPC cells. (A-D) Western blotting of the expression in apoptosis-associated proteins in (A and B) BxPC-3 cells and (C and D) YAPC cells after treatment with nectin-4-MMAE. *P<0.05 and **P<0.001.

Autophagy inhibitors enhance apoptosis induced by nectin-4-directed ADCs. Since autophagy demonstrated a crucial role in the efficacy of Nectin-4-MMAE, it was investigated if autophagy modulators could be combined to further improve efficacy. CQ (5 μM) and LY294002 (3 μM) autophagy inhibitors were chosen for testing with Nectin-4-MMAE. Cell survival was significantly lower in the combination treatment group than in the monotreatment group (Fig. 6A and B). In addition, flow cytometry results demonstrated a significant increase in the proportion of Annexin-V-positive BxPC-3 cells in the combination treatment group but not in the monotreatment group (Fig. 6C-F). Caspase-9 and PARP were more activated in the combination treatment group than in the monotreatment group, as evidence by cleaved protein bands (Fig. 7A and B). The results indicated that combining

Nectin-4-directed ADCs with autophagy inhibitors increased apoptosis and produced greater cytotoxicity.

Enhancing the antitumor efficacy of Nectin-4-directed ADC against pancreatic cancer in vivo by inhibiting autophagy. To further confirm the *in vitro* results, BxPC-3 xenograft tumor models were established to conduct *in vivo* research. Mice were randomly assigned to the negative control group, Nectin-4-MMAE group, CQ group, CQ and Nectin-4-MMAE group, and positive control drug gemcitabine group. After 21 days of drug administration, the maximum volume of the tumor was 904.74 mm^3, maximum length was 13.84 mm and maximum width was 11.73 mm. The volume of tumors in the negative control group was 741.65±82.27 mm^3, that in the gemcitabine group was 340.50±44.86 mm^3,
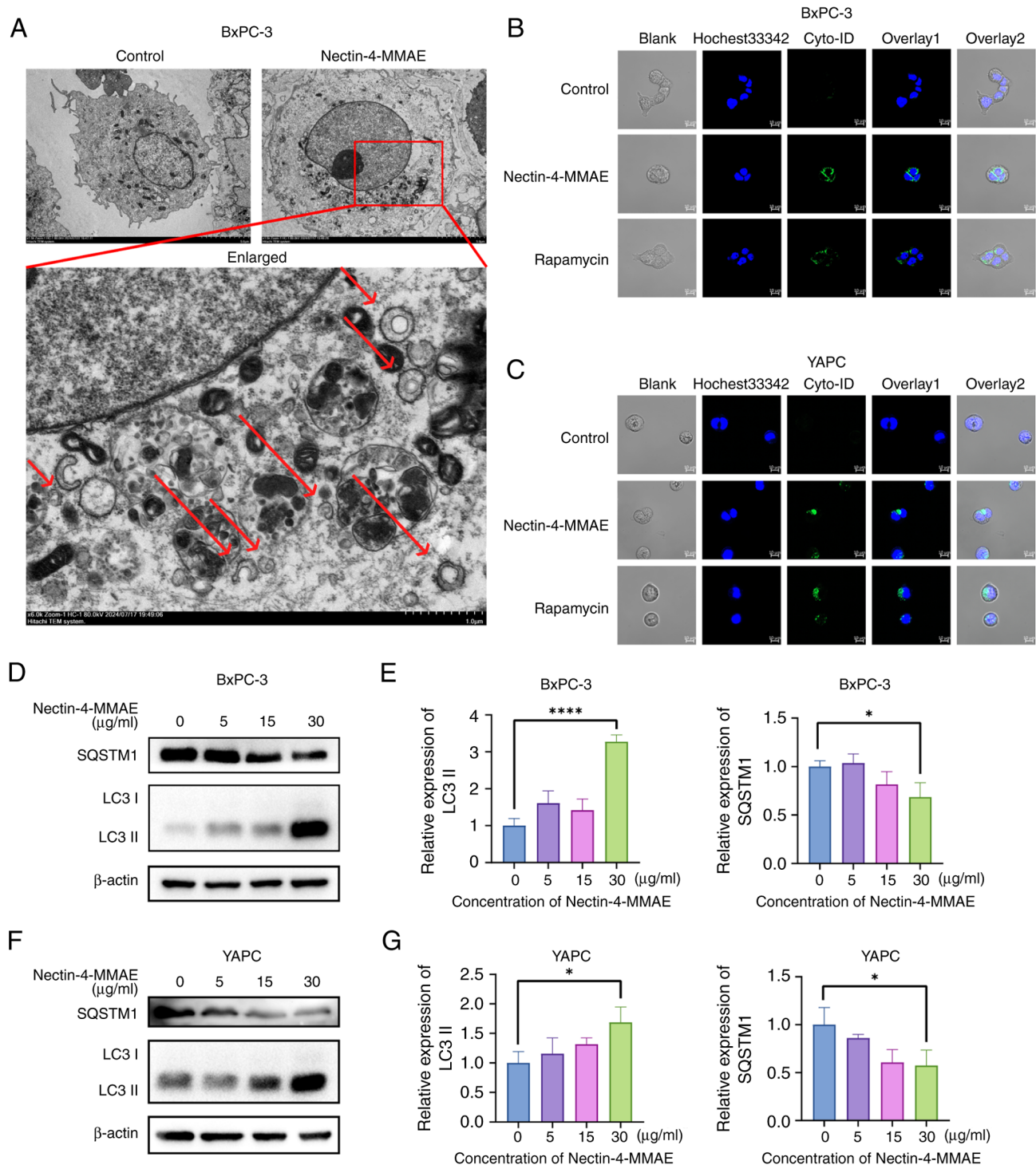
Figure 4. Nectin-4-MMAE triggers autophagy in BxPC-3 cells and YAPC cells. (A) BxPC-3 cells treated with nectin-4-MMAE were observed using electron microscopy. (B and C) Confocal microscopy was used to observe autophagosomes stained with Cyto-ID in (B) BxPC-3 cells and (C) YAPC cells. (D-F) Expression of autophagy-related proteins in (D and E) BxPC-3 and (F and G) YAPC was analyzed by western blotting. $^*P<0.05$ and $^{****}P<0.0001$.

while the tumor volume in the Nectin-4-MMAE group was 269.38±31.98 mm³, and that in the group treated with the combination of Nectin-4-MMAE and CQ was 79.12±7.62 mm³. Mice treated with Nectin-4-MMAE showed significantly reduced in tumor weights and volumes compared with the negative control group. Mice treated with CQ and Nectin-4-MMAE exhibited significantly reduced tumor weights and volumes compared with the Nectin-4-MMAE alone group (Fig. 8A and B). Tumor tissue was collected from

mice in each group, and histological changes were observed through H&E staining. The number of tumor cells was notably reduced in the Nectin-4-MMAE treatment groups, and particularly in the combined treatment group. The size of nucleoli was reduced, nuclear divisions decreased, the density of tumor cells was reduced, and the necrosis rate of the tumor increased (Fig. 8C). Furthermore, the expression levels of autophagy-related proteins in the tumor tissues of mice were detected. CQ is an inhibitor of the late stage of
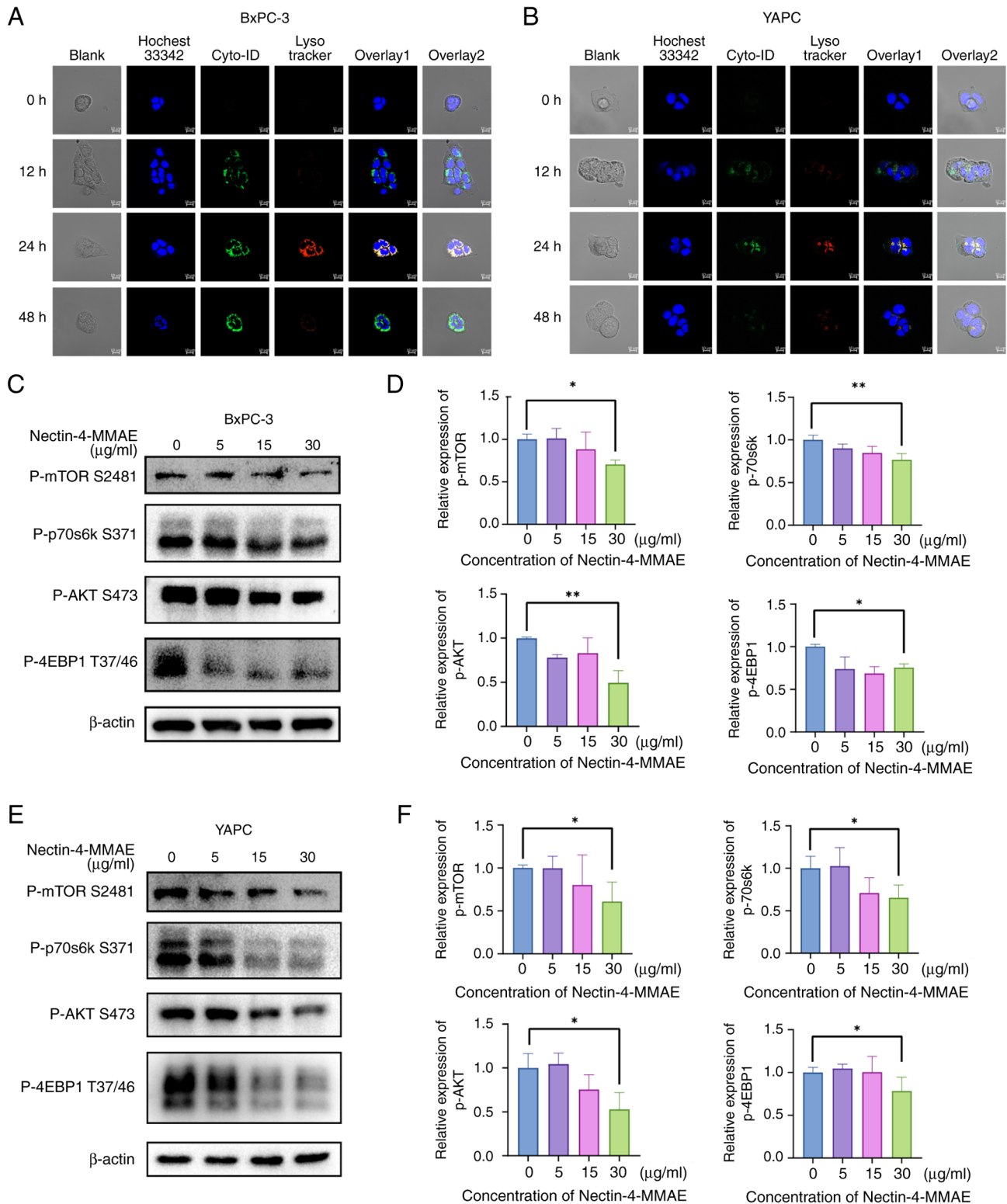
Figure 5. Nectin-4-MMAE triggers autophagic flux in BxPC-3 and YAPC cells. (A) BxPC-3 and (B) YAPC cells were incubated with Nectin-4-MMAE for the indicated time points, and stained for autophagosomes and lysosomes with Cyto-ID and Lysotracker. (C-F) Protein expression levels of p-Akt, p-mTOR, p-P70S6K and p-4EBP1 in (C and D) BxPC-3 cells and (E and F) YAPC cells after Nectin-4-MMAE treatment. *P<0.05 and **P<0.01. p-, phosphorylated.

autophagy, which can inhibit the fusion of autophagosomes and lysosomes. The degradation of LC3 II was inhibited, resulting in its accumulation (Fig. 8D). The results suggested that autophagy inhibitors promoted the treatment efficacy of Nectin-4-MMAE in animal models, which was consistent with the results of our cellular experiments.

Discussion

ADCs undergo endocytosis into target cells where they release a cytotoxic drug that causes tumor cell death (18). The cytotoxic drug MMAE in Nectin-4-MMAE promotes tubulin polymerization to perturb microtubule growth (19).
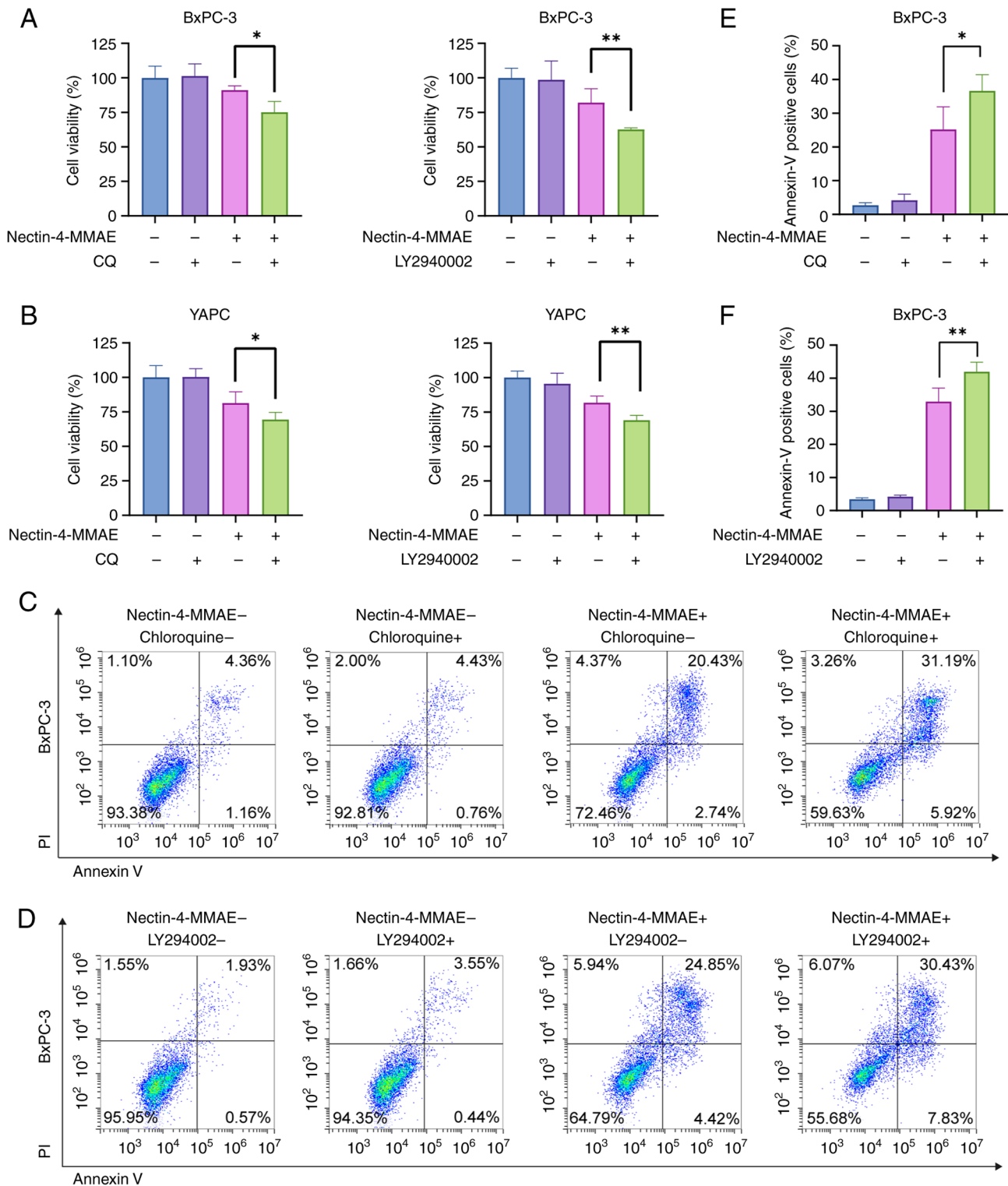
Figure 6. Autophagy inhibitors enhance Nectin-4-MMAE-induced apoptosis. (A) BxPC-3 and (B) YAPC cells were cultured in the presence of Nectin-4-MMAE, either alone or in combination with an autophagy inhibitor. Cell viability was assessed by an MTT assay. (C-F) Flow cytometric analysis of apoptosis in BxPC-3 cells. *P<0.05 and **P<0.01. CQ, chloroquine.

The specific protease caspases cause cell death by cleaving a variety of protein substrates, which are also molecular markers of apoptosis (20). The present study found that Nectin-4-MMAE demonstrated significant cytotoxicity and triggered caspase-dependent apoptosis in Nectin-4-positive pancreatic cancer cells. ADC-related research is progressing rapidly and has achieved improved therapeutic effects in the treatment of some solid tumors. Autophagy is a double-edged sword. Autophagy and apoptosis are closely related to each other, as autophagy can promote cell death in concert with apoptosis or antagonize apoptosis by promoting cell survival (21,22). The link between apoptosis and autophagy is complex and diverse, and the signals that activate autophagy usually derive from various stress conditions (23). The present
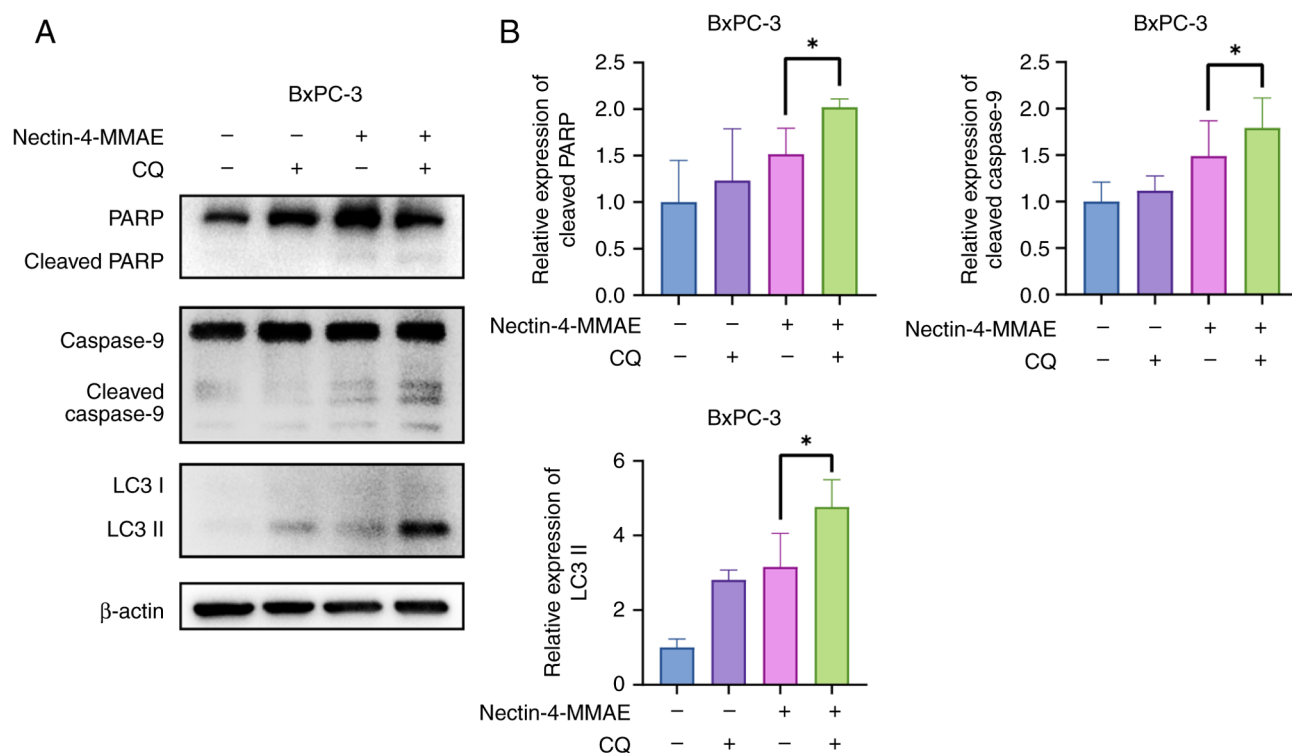
Figure 7. Autophagy inhibitors enhance nectin-4-MMAE-induced apoptosis. (A and B) Expression of cleaved PARP, cleaved Caspase-9, SQSTM1 and LC3 II protein levels in BxPC-3 cells treated with Nectin-4-MMAE and CQ were assessed using western blotting. *P<0.05. CQ, chloroquine.

study showed that Nectin-4-MMAE induced the conversion of LC3-I to LC3-II and activated autophagy in pancreatic cancer-positive cells. LC3-I is the precursor of LC3-II. LC3-II is a docking site covalently attached to cargo receptors on the membrane of phagosomes. The receptor binds to the docking site, forming a core that selectively recruits cargo. The phagosome then elongates and closes to form a separate autophagosomal compartment (24). The proximal lysosome and autophagosome then combine to produce an autophagic lysosome. In the presence of lysosomal hydrolases, the cargo is degraded and nutrients are recycled. This process was observed using electron microscopy and confocal microscopy. Xu *et al* (25,26) first reported that HuNbTROP2-HSA-MMAE significantly induced caspase-dependent apoptosis in pancreatic cancer cells and activated cytoprotective autophagy, which was consistent with the results of the present study.

The AKT/mTOR pathway is crucial to tumor development and has been implicated in the development of various malignant tumors, including pancreatic cancer. In patients with pancreatic cancer, 92% were found to have a KRAS mutation, which activates the AKT/mTOR pathway, leading to alterations in cell cycle progression and survival (27,28). mTOR consists of two multiprotein complexes, mTORC1 and mTORC2. By modifying protein synthesis, mTORC1 controls metabolism, whereas mTORC2 predominantly enhances cell survival and regulates apoptosis. mTORC1 is over-activated in a high proportion of human cancers and forms an autophagy-regulating complex that affects autophagic vesicles. Once activated, mTORC1 promotes cellular energy synthesis and metabolism by phosphorylating the downstream effectors p70S6 kinase 1 and eIF4E-binding protein (29). mTORC1 mainly promotes

energy synthesis, metabolism, autophagy inhibition and lysosome formation. mTORC2 mainly promotes cell survival and regulates apoptosis. After the BXPC-3 and YAPC cells were treated with with Nectin-4-MMAE, significantly reduced levels of p-Akt and mTOR were observed, along with the downstream proteins 4EBP1 and p70S6K. This demonstrated that Nectin-4-MMAE treatment enhanced autophagy in pancreatic cancer cells, and tumors were successfully suppressed.

Tumors disrupt overall homeostasis and biological rhythms in the body and promote tumor growth, and increased autophagy promotes cell survival in the face of environmental stresses such as nutrient deprivation (30,31). There are two ways in which malignant tumors affect body homeostasis: i) producing neurohormonal modulators that act on nerve terminal receptors after entering the circulatory system; and ii) activating circulating immune cells to regulate the function of other organs (32). Autophagy may primarily play a cytoprotective role, by maintaining energy homeostasis and nutrient requirements under starvation conditions (25,33). CQ is a late-phase autophagy inhibitor that impairs acid-dependent autophagy by increasing intra-lysosomal pH. Nectin-4-MMAE-induced autophagy can be inhibited by autophagy inhibitors such as CQ. When combined, Nectin-4-MMAE and autophagy inhibitors significantly enhanced the cytotoxic effects of Nectin-4-MMAE in the treatment of pancreatic cancer. This finding demonstrated the protective role of autophagy when treating pancreatic cancer with Nectin-4-MMAE.

Currently, the clinical application of ADCs in cancer treatment still faces numerous challenges, including off-target toxicity, drug resistance, protein aggregation and immune response. If the antibody molecules in the ADCs have poor
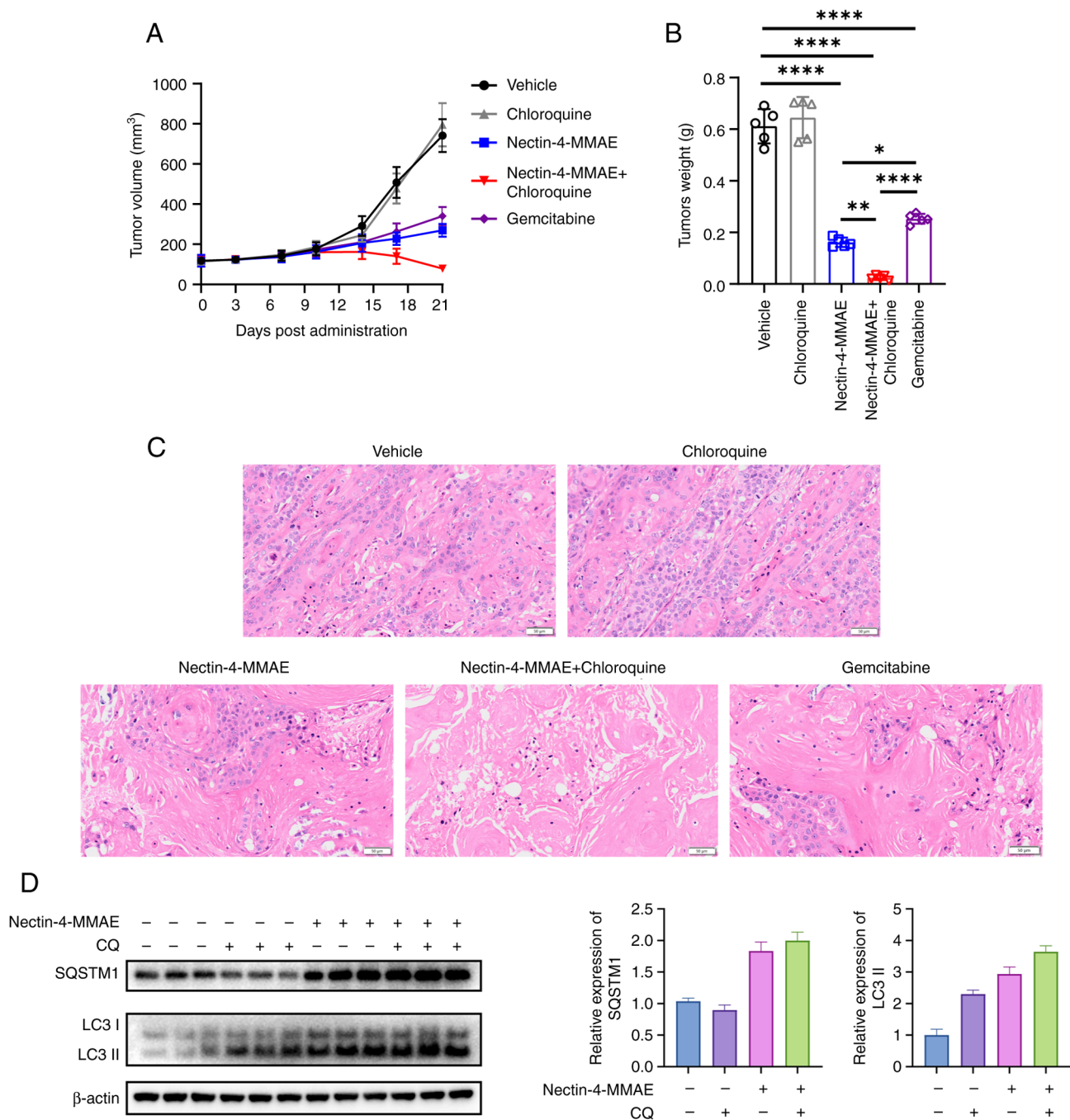
Figure 8. Nectin-4-MMAE combined with chloroquine exhibit potent therapeutic effects. (A) Tumor volumes were calculated and recorded every 3 days between the different groups. (B) The tumor weights of the different groups were recorded following treatment. The tumor weight of different groups was recorded after treatment. (C) H&E staining and immunohistochemical analysis were performed on tumor tissues from the specified groups. (D) Expression of autophagy-associated proteins in tumor tissue was assessed by western blotting. *P<0.05, **P<0.001 and ****P<0.0001.

selectivity and the target antigen exists in normal tissues, cytotoxic molecules will be delivered into normal cells, thereby causing toxic side effects. The drug resistance of human tumor tissues to ADCs is mediated by multiple factors, including alterations in the transport pathway of ADCs, downregulation of the expression of target antigens, decreased lysosomal processing capacity, overexpression of drug efflux transporters and changes in apoptotic signaling pathways (34). Drug resistance has always been a difficult problem in the treatment of tumors with ADCs. The mechanisms, causes and solutions of drug resistance still urgently need to be studied.

The present study showed that Nectin-4-MMAE exerted potent therapeutic effects on Nectin-4 positive pancreatic cancer. Notably, autophagy was activated and played a vital role in Nectin-4-MMAE-mediated therapeutic effects. Nectin-4-MMAE's therapeutic efficiency was significantly enhanced when combined with autophagy inhibitors, indicating that autophagy inhibition may be a novel approach to enhancing the ADCs' antitumor efficacy. In the future, it is expected that the therapeutic effect on pancreatic cancer will be further improved through an in-depth exploration of the specific mechanism of action of Nectin-4 in pancreatic cancer.

Availability of data and materials

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

Authors' contributions

XZ and ZD conceived the study. ZD, JX and WZ designed the experiments and proofread the manuscript. RF, CW and TY performed the experiments and prepared the figures. YX and YS analyzed the data. XZ and RF wrote the manuscript. XZ and ZD revised the manuscript. ZD and RF confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experimental procedures adhered strictly to the approved (approval no. 2025-01-SY-ZXY-02) guidelines and protocols established by the Animal Ethical Committee of the School of Pharmacy of Fudan University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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