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RESEARCH ARTICLE

ACY-241, an HDAC6 inhibitor, overcomes erlotinib resistance in human pancreatic cancer cells by inducing autophagy

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Abstract Histone deacetylase 6 (HDAC6) is a promising target for cancer treatment because it regulates cell mobility, protein trafficking, cell growth, apoptosis, and metastasis. However, the mechanism of HDAC6-induced anticancer drug resistance is unclear. In this study, we evaluated the anticancer effect of ACY-241, an HDAC6-selective inhibitor, on erlotinib-resistant pancreatic cancer cells that overexpress HDAC6. Our data revealed that ACY-241 hyperacetylated the HDAC6 substrate, α-tubulin, leading to a significant reduction in cell viability of erlotinib-resistant pancreatic cells, BxPC3-ER and HPAC-ER. Notably, a synergistic anticancer effect was observed in cells that received combined treatment with ACY-241 and erlotinib. Combined treatment effectively induced autophagy and inhibited autophagy through siLC3B, and siATG5 alleviated ACY-241-mediated cell death, as reflected by the recovery of PARP cleavage and apoptosis rates. In addition, combined ACY-241 and erlotinib treatment induced autophagy and subsequently, cell death by reducing AKT–mTOR activity and increasing phospho-AMPK signaling. Therefore, HDAC6 may be involved in the suppression of autophagy and acquisition of resistance to erlotinib in ER pancreatic cancer cells. ACY-241 to overcome erlotinib resistance could be an effective therapeutic strategy against pancreatic cancer.

Keywords Autophagy · Combination therapy · EGFR-TKI resistance · HDAC6 · ACY-241

Introduction

Pancreatic cancer is the seventh leading cause of cancer-related deaths. The mortality rate of pancreatic cancer is as high as the incident rate, and a five-year survival rate of 10% (Sung et al. 2021; Siegel et al. 2021). The epidermal growth factor receptor (EGFR) is overexpressed in pancreatic cancer, with the receptor and ligands affect the aggressiveness and severity (Cohenuram and Saif 2007). Erlotinib, an EGFR-specific tyrosine kinase inhibitor (EGFR-TKI), in combination with gemcitabine, is the standard first-line treatment for pancreatic cancer. EGFR-TKIs, such as erlotinib and gefitinib, have dramatically improved survival rates in cancer patients. However, drug resistance occurs in most patients within 1–2 years. The T790M mutation in EGFR is the most frequent among several mechanisms conferring drug resistance (Kobayashi et al. 2005). The use of second and third generation TKIs is also not the solution against drug resistance because of problems associated with resistance (Nagano et al. 2018). Therefore, overcoming drug resistance remains a key challenge in pancreatic cancer therapy.

Seong-Jun Park and Sang Hoon Joo contributed equally to the work.

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Post-translational modifications, such as acetylation and deacetylation, play roles in many cellular activities, including proliferation, differentiation, autophagy, and apoptosis (Chaudhary et al. 2019). In this context, it is reasonable to speculate that the overexpression of Histone deacetylases (HDAC) in cancer cells affects disease stage and prognosis (Witt et al. 2009). HDACs regulate gene expression by removing acetyl groups from histones and other proteins (Park and Han 2019). HDAC6, a class IIb HDAC, mainly remains in the cytoplasm and regulates the acetylation state of proteins, such as α-tubulin and histones (Zhang et al. 2015). Overexpression of HDAC6 is noticeable in cancer, and was reported to correlate with tumor progression and metastasis (Aldana-Masangkay and Sakamoto 2011). Consistently, the inhibition of HDAC6 can lead to therapeutic benefits, including inhibition of cancer cell proliferation, decrease in metastatic potential, and overcoming therapy resistance (Lee et al. 2008).

Pan-HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA, vorinostat), have been used for chemotherapy. However, their efficacy has been limited by severe adverse events when used in combination with other chemotherapeutic agents (Yoon and Eom 2016). More selective HDAC inhibitors have been developed to avoid the adverse effects related with the inhibition of class I HDACs (Bradner et al. 2010). ACY-241, a second generation HDAC6-selective inhibitor, exhibited clinical efficacy when used alone or in combination with other chemotherapies in a phase I trial (Pulya et al. 2021). ACY-241 has been reported to treat multiple myeloma and ovarian cancer by inducing cell-cycle arrest, promoting apoptosis, and inhibiting proliferation. The anticancer activity of ACY-241 looks promising, especially when used in combination with other chemotherapies (North et al. 2017; Bae et al. 2018; Ray et al. 2018; Yoo et al. 2021). Nonetheless, how ACY-241 overcomes drug resistance in pancreatic cancer is unclear.

Autophagy is an intracellular degradative process mediated by lysosomes in response to stressful conditions, such as organelle damage and starvation (Yun and Lee 2018). In mammalian cells, autophagy is largely divided into five stages: induction, vesicle nucleation, vesicle expansion, fusion, and degradation. Autophagy promotes cell survival by regulating metabolism and energy homeostasis. In cancer, autophagy affects the proliferation and death of cancer cells, with seemingly opposite roles—promotion and inhibition of cancer growth (Rosenfeldt and Ryan 2011; Tilija Pun et al. 2020). Thus, the regulation of autophagy has emerged as a new target for cancer therapy. The pharmacological inhibition of HDAC6 has been reported to regulate autophagy in various cancers, leading to cancer cell death or survival (Lee et al. 2010; Kaliszczak et al. 2018; Sharif et al. 2019). The regulation of autophagy can be exploited to increase the efficacy of chemotherapy or overcome resistance (Chang and Zou 2020). Studies on the function and role of HDAC6 in autophagy and apoptosis in EGFR-TKI-resistant cancer cells are limited.

In this study, we aimed to develop a therapy against erlotinib resistance. We observed that HDAC6 is overexpressed in erlotinib-resistant (ER) pancreatic cancer cells compared with their parental cells. We hypothesized that HDAC6 overexpression is responsible for erlotinib resistance in pancreatic cancer cells. To test our hypothesis, we investigated the role of HDAC6 by using an HDAC6 siRNA and HDAC6-specific inhibitor, ACY-241. We also evaluated whether combined treatment with ACY-241 and erlotinib could overcome erlotinib resistance in human ER pancreatic cancer cells.

Materials and methods

Chemicals and reagents

Erlotinib was purchased from LC Laboratories (Woburn, MA, USA). ACY-241 was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Dimethyl sulfoxide (DMSO) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against HDAC1, HDAC2, HDAC3, HDAC6, PARP, Ac-α-tubulin, α-tubulin, Ac-histone-H3, histone-H3, caspase-3, LC3B, p-AKT, AKT, phospho-adenosine monophosphate kinase (p-AMPK), AMPK, and mammalian target of rapamycin (mTOR) and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GAPDH and p-mTOR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and cell culture

Human pancreatic adenocarcinoma BxPC3 and HPAC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, USA) and 1% penicillin–streptomycin (HyClone Laboratories, Logan, UT, USA) in a 37 °C humidified incubator containing 5% CO_2. For maintenance, BxPC3-ER and HPAC-ER cell lines (Jang et al. 2017; Lee et al. 2017) were cultured in RPMI-1640 (10% FBS, 1% penicillin–streptomycin) with 1 μM erlotinib.

Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at 3×10^3 cells/well of a 96-well plate.

After overnight incubation, cells were treated with drugs and incubated for 48 h. Then, the cells were incubated with 20 µL MTT for 2 h. Absorbance (570 nm) was detected using the FLUOstar Omega (BMG Labtech, Germany).

Colony formation assay

BxPC3-ER and HPAC-ER cells were plated at a density of 8×10^2 cells/well of a 6-well plate. After 14 d, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), fixed with methanol for 3 min, and stained with 5% crystal violet. Colony were counted using the ImageJ software.

Western blot analysis

Western blotting has been modified and performed as described previously (Zheng et al. 2021). Cells were seeded at a density of 5×10^5 cells in a 60-mm dish. After 24 h of incubation, cells were treated with drugs, such as erlotinib or ACY-241, for 24–72 h. The cells were then lysed with cold whole-cell lysis buffer or radio-immunoprecipitation assay lysis buffer (RIPA) supplemented with Halt™ Protease, phosphatase inhibitor cocktail, PMSF, and EDTA. Proteins were quantified using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to PVDF membranes (GE Healthcare, Chicago, IL, USA). Membranes were blocked with 2.5% skim milk in TBS-T, then incubated overnight at 4 °C with primary antibodies. Membranes were washed four times with TBS-T and incubated with species-specific horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using the SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA) and developed with LAS-3000 (Fuji, Japan) according to the manufacturer's instructions.

RNA interference

Double-stranded siRNAs against human HDAC6 (NM_001321225.1), LC3B (NM_022818.4), and ATG5 (NM_001286107.1) were synthesized by Bioneer (Daejeon, Republic of Korea). Negative control siRNA was obtained from Bioneer (Cat. No.: SN-1003). Specific siRNA sequences used in this study are as follows: HDAC6 forward; 5′-CCGGUUUGCUGAAAAGGAA-3′ and reverse; 5′-UUCCUUUUCAGCAAACCGG-3′, LC3B forward; 5′-CAUAAAGACACCACUCAAA-3′ and reverse; 5′-UUUGAGUGGUGUCUUUAUG-3′, and ATG5 forward; 5′-CAGGAAAAAGAUUCCAUGU-3′ and reverse; 5′-ACAUGGAAUCUUUUUCCUG-3′. Cells were seeded at 3×10^3 cells/well of a 96-well plate or 35×10^4 cells in a 60-mm dish. After overnight incubation, the cells were transfected with siRNA or negative control siRNA using the Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfection efficacy of siRNA was measured by evaluating cell viability or through western blotting. For HDAC6 overexpression, pBJ5-HDAC6 expression vector and jetPEI (Polyplus-transfection, Illkirch, France) transfection reagent were used.

Flow cytometry

The extent of apoptosis was detected using the FITC Annexin V apoptosis detection kit with 7-AAD (BioLegend, San Diego, CA). Cells were collected after drug treatment and resuspended in binding buffer. Annexin V and 7-AAD were added to the cell suspension and the cells were incubated for 15 min at RT in the dark. Autophagy was detected using acridine orange (Thermo Scientific, Rockford, IL, USA). The cells were stained with acridine orange for 20 min and washed two times with cold DPBS, according to the manufacturer's protocol. The red fluorescence of acridine orange was quantified using the BD FACSVerse flow cytometer and BD FACSuite Software.

Immunofluorescence staining

Immunofluorescence stating has been modified and performed as described previously (Shou, J. et al. 2020). Cells were seeded at a density of 1.5×10^5 cells on the coverslips. Cells were washed once with PBS and then fixed methanol. The cells were then permeabilized using 0.1% Triton-X 100 and incubated at 4 °C for 18–20 h with LC3B antibody in a moist and humid chamber. After washing with PBS-T and incubated with an Alexa 488-conjugated secondary antibody for 1 h in the dark, nuclei were counterstained with 0.1 µg/mL DAPI. Images were then obtained by a Carl Zeiss LSM5 confocal laser microscope (Carl Zeiss, Oberkochen, Germany).

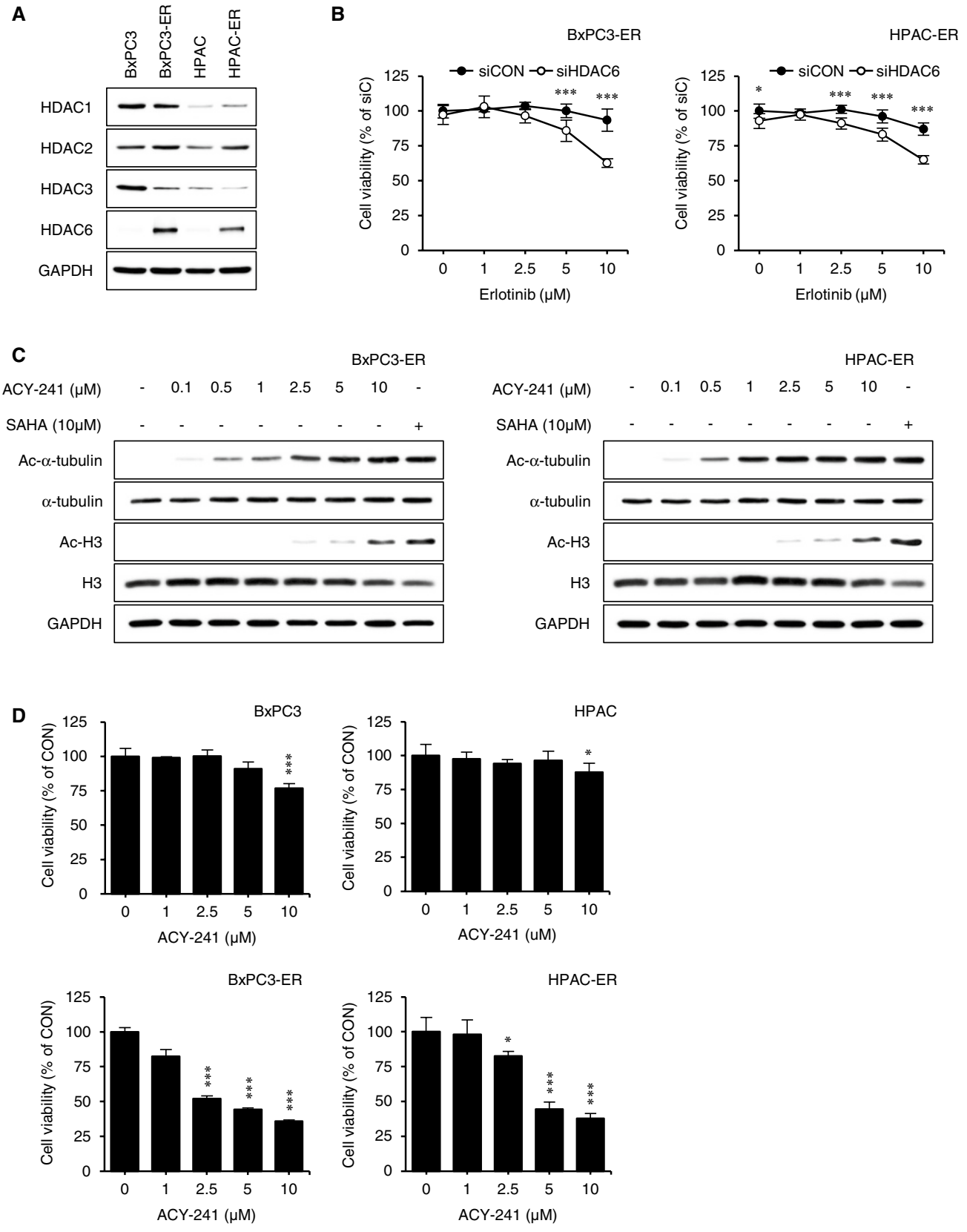
Statistical analysis

Statistical analysis was performed with Student's *t*-test or two-way ANOVA using GraphPad Prism 5. Data are presented as the mean ± standard deviation (SD).

Results

ACY-241, a selective HDAC6 inhibitor, reduces cell viability in ER pancreatic cancer cells

Previously, we established ER pancreatic cancer cells, BxPC3-ER and HPAC-ER (Jang et al. 2017; Lee et al. 2017). In this study, we compared HDAC6 expression


A
BxPC3
BxPC3-ER
HPAC
HPAC-ER
HDAC1
HDAC2
HDAC3
HDAC6
GAPDH
B
BxPC3-ER
HPAC-ER
siCON
siHDAC6
Cell viability (% of siC)
Erlotinib (μM)
C
BxPC3-ER
HPAC-ER
ACY-241 (μM)
SAHA (10μM)
Ac-α-tubulin
α-tubulin
Ac-H3
H3
GAPDH
D
BxPC3
HPAC
BxPC3-ER
HPAC-ER
Cell viability (% of CON)
ACY-241 (μM)

◂**Fig. 1** ACY-241 reduces cell viability in erlotinib-resistant (ER) pancreatic cancer cells. **A** Western blot analysis of histone deacetylases (HDACs) in erlotinib-sensitive (BxPC3 and HAPC) or resistant (BxPC3-ER and HPAC-ER) pancreatic cancer cells. **B** After knockdown of HDAC6, cells were treated with the indicated concentration of erlotinib for 48 h and cell viability was measured using the MTT assay. Statistical analysis was performed using two-way ANOVA. Error bars represent mean ± SD (n = 6). ***$p < 0.001$. **C** Cells were treated with the indicated concentration of ACY-241 for 24 h, and levels of α-tubulin, acetyl-α-tubulin, H3, and acetyl-H3 expression were analyzed using western blotting. GAPDH was used as a loading control and SAHA was used as a pan-HDAC inhibitor control. **D** After treatment with the indicated concentration of ACY-241 for 48 h, cell viability was measured using the MTT assay. Statistical analysis was conducted using a *t*-test. Error bars represent mean ± SD (n = 6). *$p < 0.05$; ***$p < 0.001$

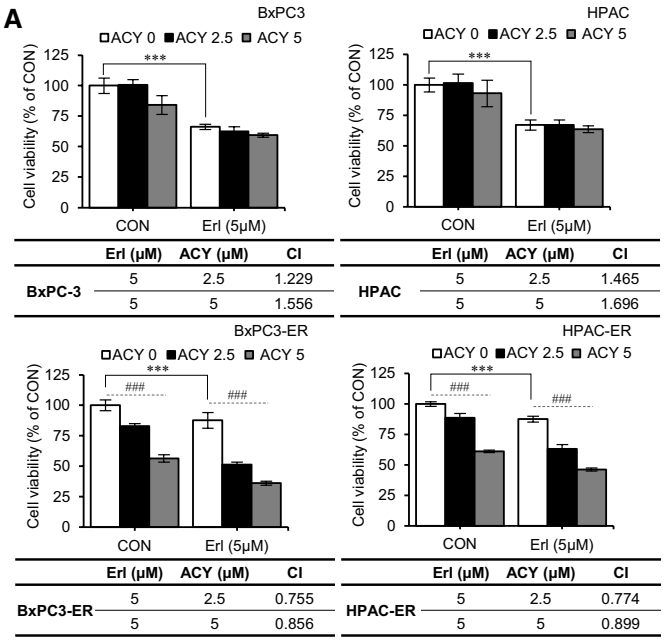
between BxPC3-ER and HPAC-ER and their parental cells, BxPC3 and HPAC, respectively. HDAC overexpression was reported to be responsible for resistance to chemotherapy (Fantin and Richon 2007). Therefore, we determined the expression levels of HDAC1, 2, and 3 (HDAC family class I) and HDAC6 (HDAC family class II) through western blot analysis. Our data revealed that HDAC6 is significantly overexpressed in BxPC3-ER and HPAC-ER cells compared with parental cells (Fig. 1A). We next investigated whether increased HDAC6 in ER cells acts as a cause of resistance to erlotinib. The results show that inhibition of HDAC6 by siRNA in both types of ER cells significantly increases susceptibility to erlotinib, implying that HDAC6 can be one of the causes of acquired resistance to erlotinib (Fig. 1B). To assess the effect of HDAC6 inhibition, we treated ER cells with ACY-241 and determined the acetylation levels of histone H3, a substrate of HDAC family class I, and α-tubulin, an HDAC6-specific substrate. Treatment with low concentration of ACY-241 caused a dramatic increase in α-tubulin acetylation but not histone H3 acetylation. This result indicates that ACY-241 might be HDAC6-specific at concentrations lower than 5 μM (Fig. 1C). Our data also showed that dose-dependent treatment with ACY-241 caused a significant decrease in cell viability of both types of ER pancreatic cancer cells, while not causing a reduction in cell viability of their parental cells. These data suggest that decreased cell viability was achieved by selectively inhibiting HDAC6 with ACY-241 (Fig. 1D).

Synergistic anticancer effects of ACY-241 combined with erlotinib in ER pancreatic cancer cells

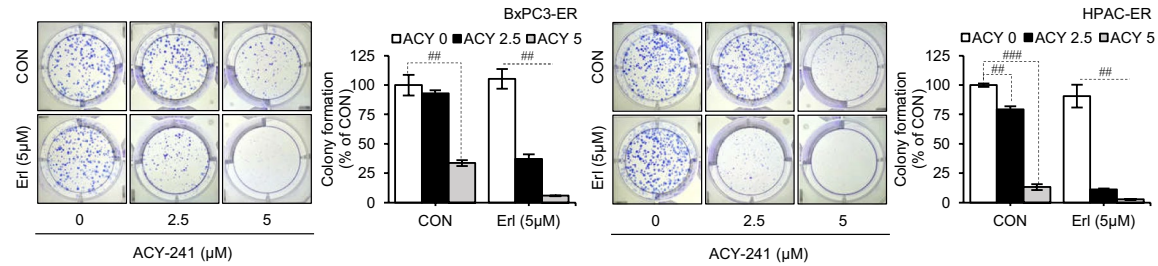
We next tested whether HDAC6 inhibition with ACY-241 could overcome erlotinib resistance. ER pancreatic cancer cells and their parental cells were treated with 5 μM erlotinib alone or in combination with 2.5–5 μM ACY-241 for 48 h. In parental cells, there was only an effect on elotinib, but no combined effect of the drugs was observed when ACY-241 and erlotinib were used together. But in ER pancreatic cancer cells, combined treatment with ACY-241 and erlotinib decreased cell viability more significantly than treatment with erlotinib alone (Fig. 2A). Consistent with cell viability data, the colony formation assay demonstrated that long-term ACY-241 treatment alone or in combination with erlotinib significantly decreased colony formation in both types of ER pancreatic cancer cells (Fig. 2B). Next, we determined the ratio of apoptotic cells after ACY-241 treatment alone or in combination with erlotinib using flow cytometry analysis. Our findings revealed that treatment with ACY-241 alone increased the number of Annexin V-positive cells, indicating apoptosis activation. Combined treatment with ACY-241 and erlotinib further increased the ratio of apoptotic cells by up to 50%, suggesting the synergism of this combination (Fig. 2C). The levels of apoptotic markers, such as cleaved caspase-3 and PARP (c-PARP), were determined by western blotting, and the result showed a clear increase of these proteins after combined ACY-241 and erlotinib treatment (Fig. 2D). Therefore, combined ACY-241 and erlotinib treatment synergistically reduces cell viability, colony formation, and induces apoptosis in ER pancreatic cell lines.

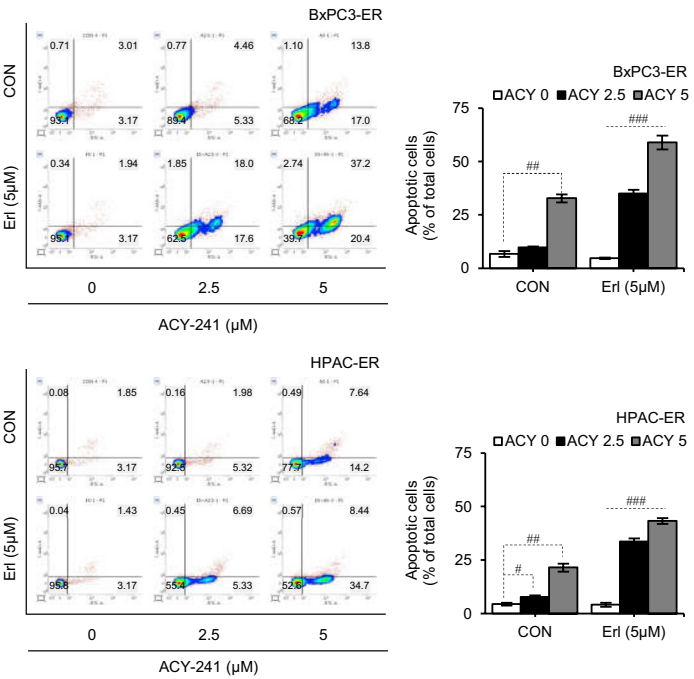
Combined ACY-241 and erlotinib treatment induces autophagy in ER pancreatic cancer cells

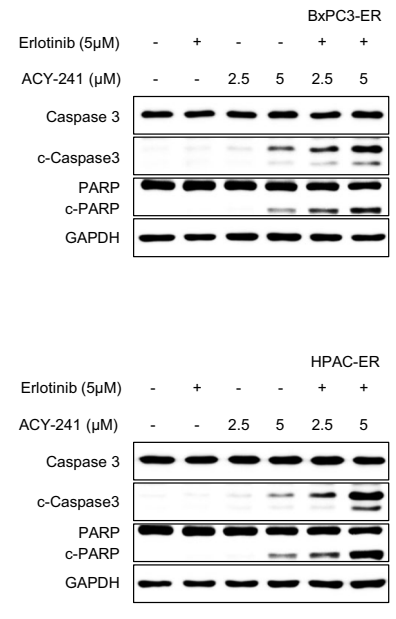
The inhibition of HDAC6 has been reported to induce various cellular responses, such as induction or inhibition of autophagy, depending on cancer cells and types of HDAC6 inhibitors (Lee et al. 2010; Kaliszczak et al. 2018; Sharif et al. 2019). Moreover, treating cells with EGFR-TKIs, such as erlotinib, can induce autophagy (Han et al. 2011; Fung et al. 2012). Based on these studies, we checked the expression of autophagy markers in both types of ER pancreatic cancer cells after treatment with erlotinib and ACY-241. ACY-241 treatment increased LC3BII levels, which increased significantly after combined ACY-241 and erlotinib treatment in both types of ER pancreatic cancer cells (Fig. 3A). Autophagy induction was also confirmed by immunofluorescence staining. Our data revealed that combined treatment with ACY-241 and erlotinib further increased LC3B expression in ER pancreatic cells (Fig. 3B). The induction of autophagy was reported to accompany an increase in acidic vesicular organelle (AVO) volume due to the increase in autolysosomes (Kanzawa et al. 2003). To determine whether ACY-241 induces autophagy in both types of ER pancreatic cancer cells, cells treated with a combination of ACY-241 and erlotinib were evaluated for the level of red fluorescence using flow cytometry. Our data revealed that dose-dependent treatment with ACY-241 increased the strength of red fluorescence. Moreover, combined ACY-241 and erlotinib treatment led to a large increase in red fluorescence (Fig. 3C). Thus, combined



	Erl (μM)	ACY (μM)	CI
BxPC-3	5	2.5	1.229
	5	5	1.556
HPAC	5	2.5	1.465
	5	5	1.696
BxPC3-ER	5	2.5	0.755
	5	5	0.856
HPAC-ER	5	2.5	0.774
	5	5	0.899







◂**Fig. 2** Combination treatment with erlotinib and ACY-241 synergistically induces an anti-cancer effect in erlotinib-resistant (ER) pancreatic cancer cells. **A** After combined treatment with erlotinib and ACY-241 for 48 h, cell viability of parental and ER cells was measured using the MTT assay. Statistical analysis was performed using two-way ANOVA. Error bars represent mean ± SD (n = 6). $^{***}p < 0.001$; $^{\#\#\#}p < 0.001$. **B** Colony formation assay. Cells were treated with a combination of erlotinib and ACY-241 for 14 days and stained with crystal violet. Representative images from three independent sets of experiments and graph quantifying colony formation. Statistical analysis was performed using two-way ANOVA. Error bars show mean ± SD (n = 3). $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. **C** Cells were treated with a combination of erlotinib and ACY-241 for 24 h, and the rate of apoptosis was measured by flow cytometric analysis with Annexin V staining (left). The quantification of apoptotic cells is shown as a graph (right). Statistical analysis was conducted using two-way ANOVA. Error bars show mean ± SD (n = 3). $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. **D** Cells were treated with a combination of erlotinib and ACY-241 for 24 h, and cleavage of caspase-3 and PARP was monitored through western blot analysis. GAPDH was used as a loading control

treatment with ACY-241 and erlotinib strongly increased the conversion of LC3BI into LC3BII, thereby inducing autophagy in both types of ER pancreatic cancer cells.

Inhibition of autophagy alleviated ACY-241-mediated apoptosis in ER pancreatic cancer cells

Autophagy and apoptosis play important roles in maintaining cellular homeostasis and are mutually regulated (Su et al. 2013). To investigate the role of autophagy on ACY-241-induced apoptosis, we silenced two autophagy related genes, LC3B and ATG5, in ER cells. The levels of cleaved PARP decreased significantly upon silencing LC3B and ATG5 (Fig. 4A, C). Consistently, flow cytometry analysis showed that knockdown of LC3B and ATG5 alleviated apoptotic cell death induced by combination treatment with ACY-241 and erlotinib (Fig. 4B, D). Thus, combination treatment with ACY-241 and erlotinib enhances apoptosis through the induction of autophagy in ER human pancreatic cancer cells.

HDAC6 might be involved in the suppression of autophagy and acquisition of resistance to erlotinib in ER pancreatic cancer cells

A study reported that defective autophagy is responsible for anticancer drug resistance and the activation of autophagy can reverse this resistance (Fung et al. 2012). In this study, we investigated how autophagy is regulated when erlotinib-sensitive and -resistant pancreatic cancer cells are treated with erlotinib. We evaluated LC3B conversion as a marker of autophagy. Erlotinib treatment led to an increase in the conversion of LC3BII in erlotinib-sensitive parental cells, whereas no significant LC3BII conversion was detected in BxPC3-ER and HPAC-ER cells. Concomitantly, erlotinib treatment led to reduced levels of p-AKT and p-mTOR and increased levels of p-AMPK in erlotinib-sensitive cells. This implies that erlotinib effectively induces autophagy by regulating upstream AKT–mTOR and AMPK signaling in erlotinib-sensitive cells. However, in ER pancreatic cancer cells, erlotinib did not induce autophagy and had no significant effect on these upstream signaling pathways (Fig. 5A). Based on this observation, we speculated that resistance to erlotinib can be caused by failure of autophagy induction. Therefore, we investigated the correlation between the failure to induce autophagy by erlotinib and HADC6 overexpression in ER cell lines. We used siRNA to knockdown HDAC6, the target of ACY-241, and then identified how it affected autophagy after erlotinib treatment in ER cells. Erlotinib treatment induced LC3B conversion in HDAC6 siRNA-treated ER cells through a decrease in p-AKT and p-mTOR and an increase in p-AMPK levels, while little change in LC3B conversion in control siRNA-treated ER cells (Fig. 5B). Conversely, HDAC6 overexpression in erlotinib sensitive-BxPC3 cells has been shown to reduce LC3B conversion increased by erlotinib treatment (Supplementary Figure). In addition, combined ACY-241 and erlotinib treatment strongly increased LC3BII conversion through the regulation of same upstream signaling pathways in both types of ER pancreatic cancer cells (Fig. 5C). Therefore, HDAC6 overexpression in ER pancreatic cancer cells might inhibit erlotinib-induced autophagy, thereby leading to erlotinib resistance. However, inhibition of HDAC6 by siRNA or ACY-241 led to erlotinib-induced autophagy and overcoming of erlotinib resistance in ER pancreatic cancer cells (Fig. 5D).

Discussion

Although various therapies have been developed to treat pancreatic cancer, drug resistance remains a challenge (Zhang et al. 2020). Combination therapy has emerged as a promising strategy to overcome drug resistance and the complexity of signaling pathways (Chen et al. 2014). In addition to EGFR-TKI, other drugs can be combined to inhibit other receptors, such as VEGFR, FGFR, and MET. Inhibitors of downstream signaling, such as PI3K–Akt–mTOR, Ras–Raf–MEK–ERK, and JAK–STAT, can be used in addition to EGFR-TKI treatment to overcome this resistance (Tong et al. 2017).

HDAC is a promising target for both single and combined treatments against cancer. HDAC inhibitors regulate the expression of various genes by modulating the acetylation states of histone protein (Eckschlager et al. 2017). Pan-HDAC inhibitors (panobinostat, belinostat, and vorinostat) and HDAC class I inhibitors (tucidinostat and mocetinostat) have been tested in clinical trials for treating various blood

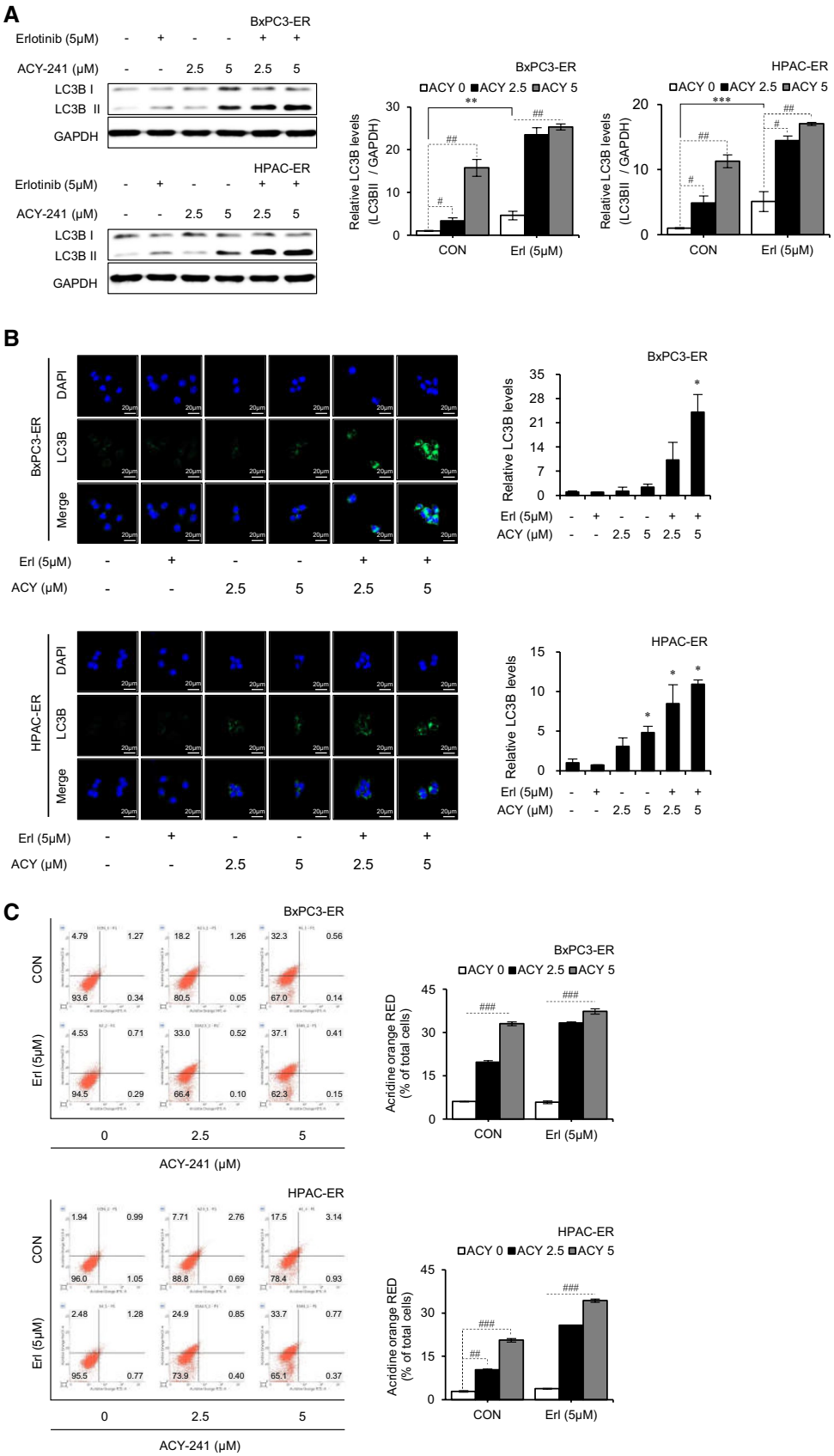


Fig. 3 Combination treatment with erlotinib and ACY-241 induces autophagy in erlotinib-resistant (ER) pancreatic cancer cells. **A** After treatment with a combination of erlotinib and ACY-241 for 24 h, levels of LC3B were measured using western blot analysis. GAPDH was used as a loading control. Quantitation of protein intensity was performed using the ImageJ software. Statistical analysis was performed using two-way ANOVA. Error bars represent mean ± SD (n = 3). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. **B** Immunofluorescence staining of LC3B. Cells were treated with a combination of erlotinib and ACY-241 for 24 h. LC3B-positive cells were detected via fluorescence microscopy, and intensity was quantified using the ImageJ software. Statistical analysis was conducted using Student's *t*-test. Error bars represent mean ± SD (n = 3). $^{*}p < 0.05$. **C** Cells were treated with a combination of erlotinib and ACY-241 for 24 h. Acridine orange staining was performed to identify autophagic cells through FACS. Statistical analysis was performed using two-way ANOVA. Error bars represent mean ± SD (n = 3). $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$

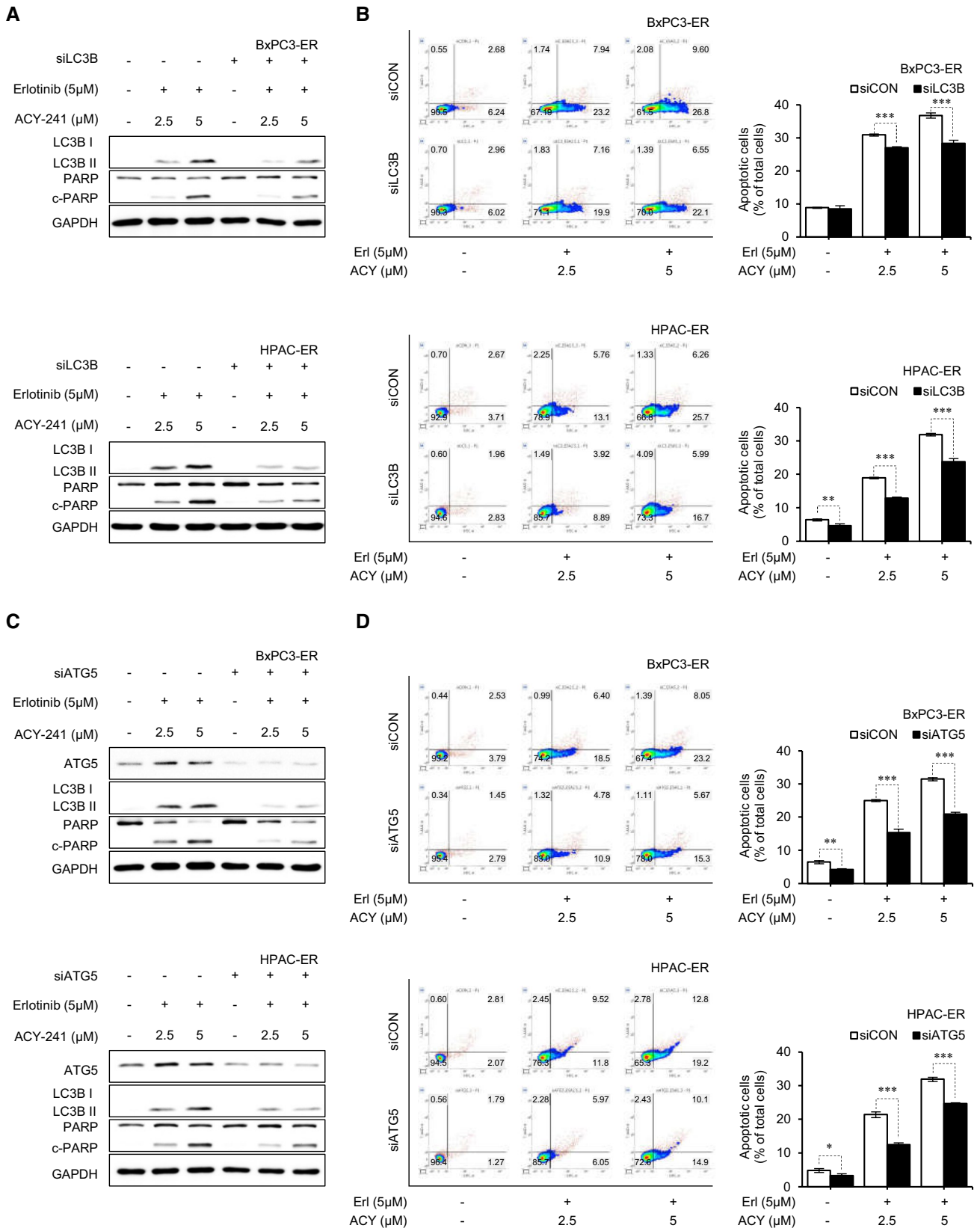


Fig. 4 Inhibition of autophagy alleviates apoptosis in erlotinib-resistant (ER) pancreatic cancer cells. **A, C** Cells were transfected with siLC3B or siATG5 and treated with a combination of erlotinib and ACY-241 for 24 h. Levels of LC3B, ATG5, and PARP expression were measured using western blot analysis. GAPDH was used as a loading control. **B, D** Flow cytometry analysis was performed to check the effect of siLC3B or siATG5 on apoptosis of ER cells. Statistical analysis was performed using two-way ANOVA. Error bars represent mean ± SD (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

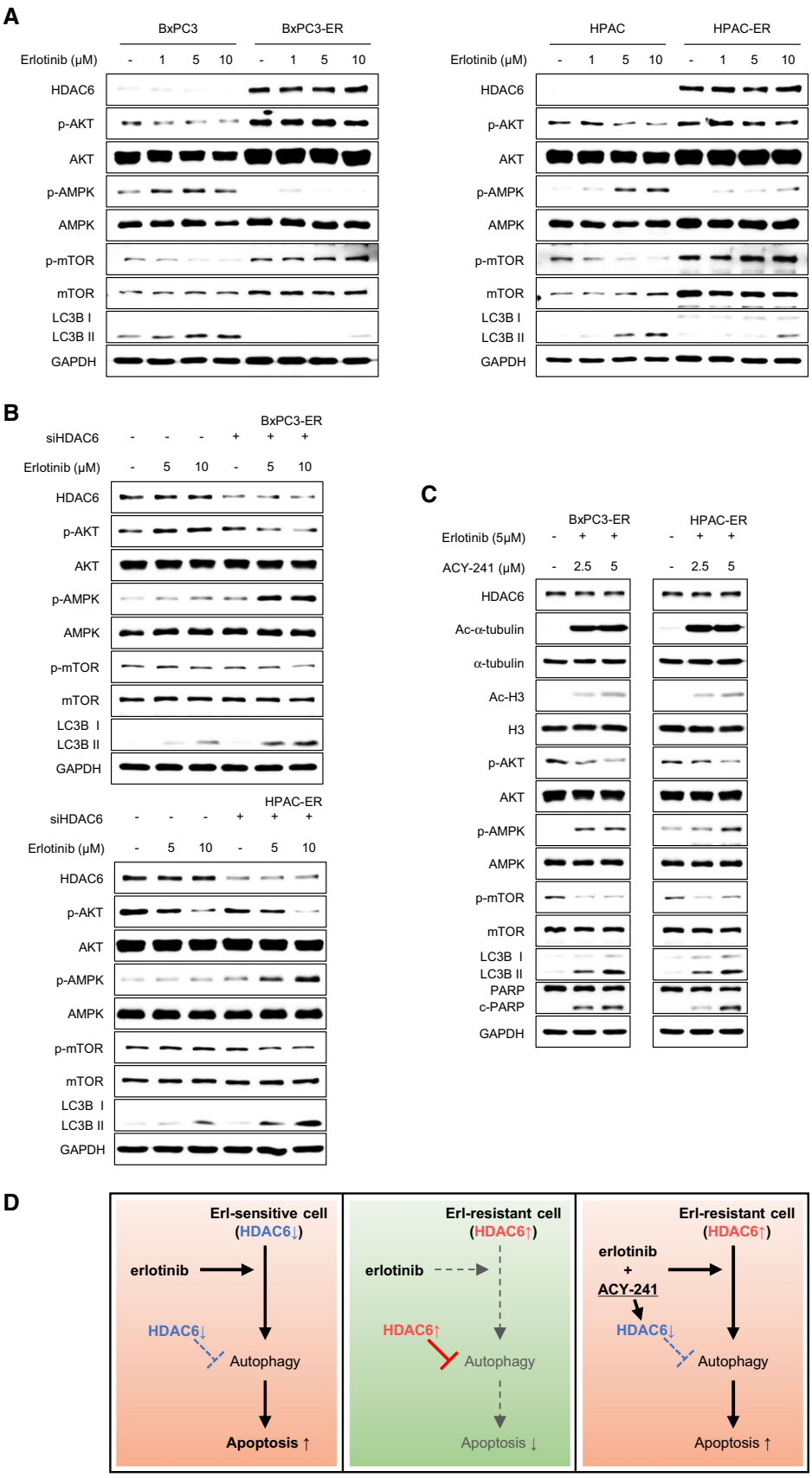


Fig. 5 The role of histone deacetylase (HDAC) 6 in suppressing autophagy and acquiring erlotinib resistance in erlotinib-resistant (ER) pancreatic cancer cells. **A** Erlotinib-sensitive (BxPC3 and HAPC) or resistant (BxPC3-ER and HPAC-ER) pancreatic cancer cells were treated with the indicated concentration of erlotinib for 24 h and level of protein expression was quantified using western blot analysis. GAPDH was used as a loading control. **B** Cells were transfected with siHDAC6 and treated with the indicated concentration of erlotinib for 72 h. Level of protein expression was measured using western blot analysis. **C** Cells were treated with a combination of erlotinib and ACY-241 for 24 h and level of protein expression was quantified using western blot analysis. GAPDH was used as a loading control. **D** Proposed mechanism of the anticancer effect of ACY-241 and erlotinib in erlotinib-sensitive and resistant pancreatic cancer cells

cancers (West and Johnstone 2014). HDAC inhibitors, romidepsin and MPT0E028, were reported to increase sensitivity to erlotinib in NSCLC (Zhang et al. 2009; Chen et al. 2013). A phase I trial is under way as vorinostat has been reported to induce apoptosis when combined with gefitinib (Nakagawa et al. 2013). Combined treatment with several pan-HDAC inhibitors and EGFR-TKIs can induce autophagic cell death in T790M mutant lung cancer (Lee et al. 2015). However, the clinical application of pan-HDAC inhibitors or HDAC class I inhibitors is limited, because they lack specificity and exhibit cytotoxicity when administered alone or in combination with other drugs (Deubzer et al. 2013; Koutsounas et al. 2013; Yoon and Eom 2016). Moreover, few studies have evaluated the use of HDAC inhibitors in treating pancreatic cancer. Therefore, we used ACY-241, a selective HDAC6 inhibitor, to minimize the side effects while increasing the effectiveness of a pan-HDAC inhibitor.

Autophagy is induced by AMPK, a key energy sensor that regulates metabolism to maintain energy homeostasis, and inhibited by mTOR, which integrates the upstream signaling of class I PI3K/AKT and AMPK (Kim et al. 2011). The absence of growth signals leads to the deactivation of AKT and accumulation of tuberous sclerosis protein 1/2 (TSC1/2), which inactivates mTOR by activating Ras homolog enriched in brain (Rheb) (Efeyan et al. 2013). Then, inactivated mTOR activates the Unc-51-like kinase (ULK) complex to induce autophagy (Li et al. 2020). When energy levels are low in cells, AMPK first activates TSC1/2, and then, inactivates mTOR and induces autophagy (Lacher et al. 2010). In general, autophagy induces drug resistance by protecting cancer cells from chemotherapy. The upregulation of autophagy can prevent DNA damage (White 2016) or increase drug resistance by increasing the expression of multidrug resistance (MDR) genes (Zhang et al. 2016). However, persistent and excessive autophagy can cause apoptotic cell death. Thus, the induction of autophagy by diverse therapeutic stresses has a dual role in promoting cell survival or death depending on cell type and environmental stress (Chang and Zou 2020). Baicalein, a flavonoid with anticancer properties, can induce autophagic cell death by activating AMPK/ULK1 and downregulating mTORC1 (Aryal et al. 2014). Pharmacological induction of autophagy in an EGFR-TKI-resistant cancer cell line was shown to increase the sensitivity of EGFR-TKI and induce autophagic cell death through the AKT–mTOR pathway (Fung et al. 2012).

HDAC6, a class IIb HDAC, is overexpressed in cancer and associated with increased tumorigenesis and cell survival (Aldana-Masangkay and Sakamoto 2011). Unlike that for other HDACs, knocking out HDAC6 in mice does not exhibit cytotoxicity, which is why active research is being conducted on HDAC6 as a target for cancer therapy (Zhang et al. 2008). Studies have shown that HDAC6 might regulate autophagy and cell death through various mechanisms. For example, C1A, an HDAC6 inhibitor, was reported to reduce cancer cell viability by blocking the fusion of autophagosomes and lysosomes in myc-positive neuroblastoma, KRAS-positive colorectal cancer, and multiple myeloma cells (Kaliszczak et al. 2018). In multiple myeloma, HDAC6 affects aggresome formation in cells; treatment with ACY-1215, an HDAC6 inhibitor, inhibits aggresome formation, which causes inhibition of autophagy and subsequently, cell death (Mishima et al. 2015). By contrast, treatment with another HDAC6 inhibitor, J22352, can induce autophagic cell death in glioblastoma (Liu et al. 2019). These are conflicting reports showing that HDAC6 inhibition by different inhibitors can induce apoptosis by inhibiting or promoting autophagy. Therefore, we tried to establish the relationship between autophagy and apoptosis in human pancreatic cancer cells, where HDAC6 is overexpressed. Although phase I/II clinical trials using ACY-1215, a commonly used HDAC6-selective inhibitor, have been conducted, the use of ACY-1215 in the clinic is a challenge owing to its low absorption (Hideshima et al. 2016). Therefore, we used ACY-241, a second-generation HDAC6-selective inhibitor that overcomes these shortcomings and increases selectivity for HDAC6 (Yoo et al. 2021). Our findings revealed that ACY-241 alone increased LC3B-II conversion; combined treatment with ACY-241 and erlotinib induced autophagy more strongly by regulating AKT–mTOR and AMPK signaling downstream.

Although extensive studies have been conducted on autophagy regulation through mTOR and AMPK signaling, studies on how HDAC6 controls autophagy to contribute to drug resistance have not been conducted. In this study, we observed increased expression of HDAC6 in two types of ER pancreatic cancer cells compared with their parental cells. We hypothesized that HDAC6 overexpression correlates with the acquisition of erlotinib resistance. Our results reveal several important points. First, HDAC6 is overexpressed in ER pancreatic cancer cells compared with parental cells. Second, erlotinib reduces cell viability by inducing autophagy in parental cells, but not in ER cell lines. Thus, the induction of autophagy by erlotinib can act as a prerequisite for cell death. Third, sensitivity to erlotinib in ER cells was synergistically enhanced when HDAC6 activity was inhibited using siRNA or a selective inhibitor. Fourth, combined ACY-241 and erlotinib treatment induced autophagic cell death by regulating AKT–mTOR and AMPK signaling. Therefore, whether autophagy is induced by erlotinib is one of the causes for the acquisition of erlotinib resistance and HDAC6 negatively regulates induction of autophagy in this process. In addition, autophagy can be induced by the selective inhibition of HDAC6, which can help overcome erlotinib resistance in ER pancreatic cancer cells. Consistent with this, the emerging data suggest the role of HDAC as a resistance regulator in EGFR-TKI-resistant cancer cells by

showing that inhibition of HDAC sensitizes these resistant cells to EGFR-TKIs (Wang et al. 2016; Yu et al. 2017). However, there are no reports yet on how and why HDAC6 can be increased with the emergence of acquired resistance to erlotinib. We are still unaware of the answer to this question and will need to investigate how HDAC6 can be a new regulator of acquired drug resistance through follow-up studies.

Taken together, our findings suggest that ACY-241, a selective HDAC6 inhibitor, has anticancer effects in ER pancreatic cancer cell lines and combined ACY-241 and erlotinib treatment synergistically induces cell death in ER cancer cells. In addition, HDAC6 is implicated in the suppression of autophagy and acquisition of erlotinib resistance in ER pancreatic cancer cells. Therefore, combined ACY-241 and erlotinib treatment could be an effective therapeutic strategy against pancreatic cancer.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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