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AMPK-mTOR-ULK1 axis activation-dependent autophagy promotes hydroxycamptothecin-induced apoptosis in human bladder cancer cells

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Abstract

10-hydroxycamptothecin (HCPT), a natural plant extract, exerts anticancer capacity. HCPT has been reported to induce apoptosis and autophagy in human cancer cells. The interaction between autophagy and apoptosis induced by HCPT and the molecular mechanism in bladder cancer cells were investigated in this study. Our results confirmed that HCPT suppressed cell viability and migration and caused cell-cycle arrest in T24 and 5637. Then, we used Z-VAD(OMe)-FMK to clarify that apoptosis induced by HCPT was mediated by caspase. Moreover, HCPT boosted autophagy through activating the AMPK/mTOR/ULK1 pathway. Blocking autophagy by 3-methyladenine, the adenosine monophosphate-activated protein kinase (AMPK) inhibitor dorsomorphin and siATG7 reversed HCPT-induced cytotoxicity. Conversely, rapamycin and the AMPK activator AICAR enhanced growth inhibition and cell apoptosis, suggesting that autophagy played a proapoptosis role. Taken together, our findings showed that HCPT-induced autophagy mediated by the AMPK pathway in T24 and 5637 cell lines, which reinforced the apoptosis, indicating that HCPT together with autophagy activator would be a novel strategy for clinical treatment in bladder cancer.

KEYWORDS
AMPK pathway, apoptosis, autophagy, bladder cancer, hydroxycamptothecin

1 | INTRODUCTION

Bladder cancer is the ninth most common malignancy in the world (Ferlay et al., 2015; Miller et al., 2016) and the second most common malignancy in the urinary system (Malats & Real, 2015). About 75% of new-onset bladder cancer patients are nonmuscular invasive bladder cancer (NMIBC) (Casey et al., 2015; Chavan, Bray, Lortet-Tieulent, Goodman, & Jemal, 2014), who were divided into three classes (low, medium, and high risk) on the basis of their different conditions (Jarvinen et al., 2015). Unfortunately, about a third of noninvasive bladder cancer patients turn into MIBC with higher risks for recurrence, progression, and mortality (Sun & Trinh, 2015). Currently, the standard treatment for NMIBC is transurethral cystectomy (TURBT). The European association of urology (EAU) and American urological association (AUA) guidelines recommended that all patients with NMIBC should receive intravesical instillation therapy immediately after TURBT (Jarvinen et al., 2015; Kaufman, Shipley, & Feldman, 2009). Timely, sufficient and regular intravesical instillation could prevent the recurrence and progression of the disease (Cambier et al., 2016). But, due to the limited instillation time and biological penetrability, the long-term curative effect is poor, which stresses doctors and patients (Lu, Neoh, Kang, Mahendran, & Chiong, 2015), thus therapeutic strategies and further studies are urgently needed.

10-hydroxycamptothecin (HCPT) is a natural plant extract isolated from Chinese tree Camptotheca acuminate, which is a derivative of CPT with a specific 10-hydroxy group. Similar to CPT, HCPT has been verified to stabilize the shearing complex between DNA and topoisomerase via inhibiting DNA topoisomerase I, which prevents the connection with DNA and ultimately leads to irreversible DNA damage. However, because of the 10-hydroxy group, HCPT is less toxic than CPT (Capranico, Marinello, & Chillemi, 2017; Wall & Wani, 1996; Wang et al., 2019; Yin et al., 2013). HCPT has been widely used in clinical practice for decades, including intravesical instillation in bladder cancer (Urasaki, Takebayashi, & Pommier, 2000; G. Zhang et al., 2011; R. Zhang et al., 1998). Numerous clinical trials confirmed that HCPT could be a first-line treatment in various human malignant tumors (Arnold et al., 2010; Han et al., 2014; Pu et al., 2009). Previous basic research demonstrated that HCPT remarkably induced apoptosis, autophagy and cell-cycle arrest in different human cell lines, which confirmed its broad-spectrum cytotoxicity (Bian et al., 2015; P. Chen, Liu, & Hu, 2013; Fei, Chi, & Weng, 2013; Liu, Zhu, Getzenberg, & Veltri, 2015; Nie et al., 2015; Wang et al., 2019; Yin et al., 2013; Zaki, 2014; Y. Zhang et al., 2013). However, the exact molecular biological mechanism and pathway of HCPT working upon bladder cancer cells remain mysterious.

Autophagy, a dynamic and continual biological behavior, plays a significant role in many biological processes. It prevents misaggregation and misfolding of proteins to maintain intracellular homeostasis (Baehrecke, 2005; Mizushima, Yoshimori, & Levine, 2010). Autophagy starts from the double-membrane vacuoles, which contained aging organelles, or portions of the cytoplasm and foreign agents, and finally they are fused with the lysosome to be broken down by hydrolytic enzymes (He & Klionsky, 2009). Inhibition of autophagy could induce oxidative stress through the ROS/HO-1 pathway and promote oncogenesis (Mathew et al., 2007, 2009). In contrast, because of disordered biological behaviors such as proliferation, invasion, and migration along with increased need for nutrients, autophagy appears to be more important and indispensable for tumor cells to improve the utilization efficiency (Rabinowitz & White, 2010). Autophagy can not only causes autophagic cell death (a kind of nonapoptotic programmed cell death) to suppress the occurrence and development of malignancy but also maintain cellular homeostasis when undergoing intracellular and extracellular stresses and makes more tumor cells survive after pharmacotherapy, chemotherapy, or radiation. Tumor type, pathological stage, clinical stage, genetic factors, and microenvironment are associated with autophagy to a certain degree, and this is enigmatic to researchers (Kang, Zhang, Zeh, Lotze, & Tang, 2013; Kubisch et al., 2013). The autophagy regulating pathway is not unique, but complicated. In glioma cells, autophagy is induced by cobalt chloride mainly through the p53 pathway (B.-C. Cheng et al., 2017). Another study found that drug-induced MAPK signaling pathway activation might initiate protective autophagy (Utaipan, Athipornchai, Suksamrarn, Chunsrivirot, & Chunglok, 2017). Among various autophagy-related signaling pathways, AMPK, a kinase sensing the energy or nutrient state of cells, has always been a hotspot (Hardie, 2007).

In our study, the cytotoxic and autophagic effects of HCPT on bladder cancer cells were investigated initially. Furthermore, we confirmed that activation of AMPKα (Thr172), suppressing mTOR and phosphorylation of ULK1 (Ser555) participated in HCPT-induced autophagy and apoptosis. We also revealed that autophagy enhanced apoptosis. Overall, our research demonstrated that HCPT-induced autophagy plays a cytotoxic role in T24 and 5637, and these data suggested that HCPT together with autophagy activators might be a brand-new strategy for bladder cancer therapy.

2 | MATERIALS AND METHODS

2.1 | Cell culture

T24 and 5637 cell lines were purchased from the Chinese Academy of Sciences. 1640 medium was used to culture the cells and supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37°C.

2.2 | Reagents and antibodies

HCPT, Dorsomorphin and Acadesine (AICAR) were products of Selleck Chemicals (Huston, TX). Hoechst 33342 powder and cell counting kit-8 (CCK-8) were general gifts from Mengjing Fan. The Apoptosis Detection Kit #556547 was bought from BD (San Jose, CA). The ATG7 siRNA kit (SIGS0005319-1) was a product of RIBOBIO (Guangzhou, China). Antibodies against Bcl-2 (ab182858), Bax (ab32503), LC3B (ab192890), mTOR (ab2732), p-mTOR (Ser2448), AMPKα (ab32047) and ULK1 (ab128859) were bought from Abcam (San Francisco, CA). Antibodies against β-actin (20536-1-AP) were from Proteintech (Chicago, IL). Antibodies against cleaved poly(ADP-ribose) polymerase (PARP) (9541), cleaved caspase 3 (9664), SQSTM1/p62 (8025), p-AMPKα (2535), p-ULK1 (5869) were products of Cell Signaling Technology (Beverly, NJ).

2.3 | Cell viability assay

A 96-well plate was used for CCK-8 assay. Ten thousand of cells per well were cultured with 1640 medium (10% FBS), then treated with different reagents. After treatment, 10 μl CCK-8 was added to each well and incubated for 2.5 hr at 37°C. The cell viability was determined using a microplate reader (Bio-Rad, CA) at 450 nm.

2.4 | Hoechst 33342 analysis

The cells were stained after 24 hr HCPT (0.5 or 1 μM) treatment with Hoechst 33342 for 10 min. Afterwards, the samples were rinsed with phosphate-buffered saline (PBS) twice and observed under fluorescence microscopy.

2.5 | Wound-healing assay

The cells were seeded in six-well plates to reach 80–90% confluence. Pipette tips were used to create a linear wound. Then, the cells were

incubated without serum with HCPT for another 24 hr. We observed and took photos at 0, 24 hr using Nikon microscopy (Nikon, Tokyo, Japan), and the migration rate of cells was measured by ImageJ (Bethesda).

2.6 | Transwell assay

The cells (8×10^4 cells/ml) were added to the upper chamber and cultured with serum-free 1640 medium. The lower chamber was a chemo-attractant containing 10% FBS 1640 medium. After 48 hr, the cells remaining in the upper chamber were removed with a cotton swab. Afterwards, migrated cells at the lower membrane surface were fixed with 4% paraformaldehyde for 10 min and stained with crystal violet. The following observation was under a microscope (Nikon) using a ×400 objective.

2.7 | Colony formation assay

One thousand of cancer cells per well were seeded into a six-well plate. Then, the cells were cultured with fresh medium containing HCPT or dimethyl sulfoxide (DMSO). The medium wase changed every 3 days. Twelve days later, colonies (consisting of >50 cells) were fixed for 10 min followed by crystal violet staining at room temperature.

2.8 | Flow cytometry for apoptosis analysis

Trypsin-treated cells were re-suspended and then stained with fluorescein isothiocyanate (FITC) and propidium iodide (PI) in 400 μl binding buffer. The samples were incubated for 15 min at room temperature and the apoptosis rate was determined by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson).

2.9 | Cell-cycle analysis

Cell-cycle analysis was determined by flow cytometry (FACSCalibur; Becton Dickinson) and CellQuest software (Becton Dickinson). Cell-cycle staining buffer (Multi Sciences, Hangzhou, China) was used to detect the distribution of cell cycle.

2.10 | Confocal microscopy observation

Cells pretransfected with lentivirus carrying mCherry-GFP-LC3B were seeded into a glass-bottom culture dish overnight. The cells were cultured in different conditions for another 24 hr. Then, the cells were washed with PBS and observed under a confocal microscope (FV1000; Olympus, Tokyo, Japan). The numbers of auto puncta were counted by images obtained with an OLYMPUS FLUOVIEW (Olympus).

2.11 | RNA interference of ATG7

When cells reached 30% confluency, ATG7 small interfering RNA (siRNA) or control siRNA were diluted in buffer and reagent to transfect for 48 hr. Afterwards, the medium was replaced and the transfected cells used for the following studies.

2.12 | Western blot analysis

Briefly, total proteins were extracted by using RIPA and this was followed by protein concentration measurement. Proteins (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We transferred the protein to polyvinylidene fluoride (PVDF) membranes, incubating the membrane with 5% de-fat milk at room temperature for about 1 hr. After rinsing the PVDF membrane with TBST, it was incubated with primary antibodies at 4℃ overnight and secondary antibody at 37℃ for 2 hr. AN ECL detection system was used to detect the bands. ImageJ was used to calculated the intensities of the bands.

2.13 | Transmission electron microscopy (TEM)

The cells with different treatment were fixed by 2.5% glutaraldehyde containing sodium cacodylate in 4℃ for at least 6 hr. We fixed the sample with 1% osmium tetroxide and then dehydrated. The samples were embedded and made into 50 nm sections and stained with 3% uranyl acetate and lead citrate. The ultrastructure of cells was observed and obtained under the TEM (H-7650; HITACHI, Tokyo, Japan).

2.14 | Statistical analysis

All data were listed and analyzed as the mean ± standard deviation (SD). Differences between groups were analyzed in SPSS (New York) by using one-way or two-way analysis of variance. A $p < .05$ at two-tailed was regarded as statistically significant in this study.

3 | RESULTS

3.1 | HCPT suppressed viability, proliferation, migration and caused G2/M phase arrest of human bladder cancer cells

To demonstrate the effects on human bladder cell viability, migration, proliferation, and cell cycle distribution, T24 and 5637 cell lines were cultured with HCPT for 24 hr. The following CCK-8 assay showed that HCPT suppressed cell viability and proliferation in T24 and 5637 in a dose-dependent manner (Figure 1a). Furthermore, the wound-healing and transwell assay showed that HCPT significantly decreased the migrated cells (Figure 1b,c). Colony formation assay indicated that HCPT suppressed colony formation (Figure 1d). HCPT also induced G2/M phase arrest (Figure 1e). According to the above

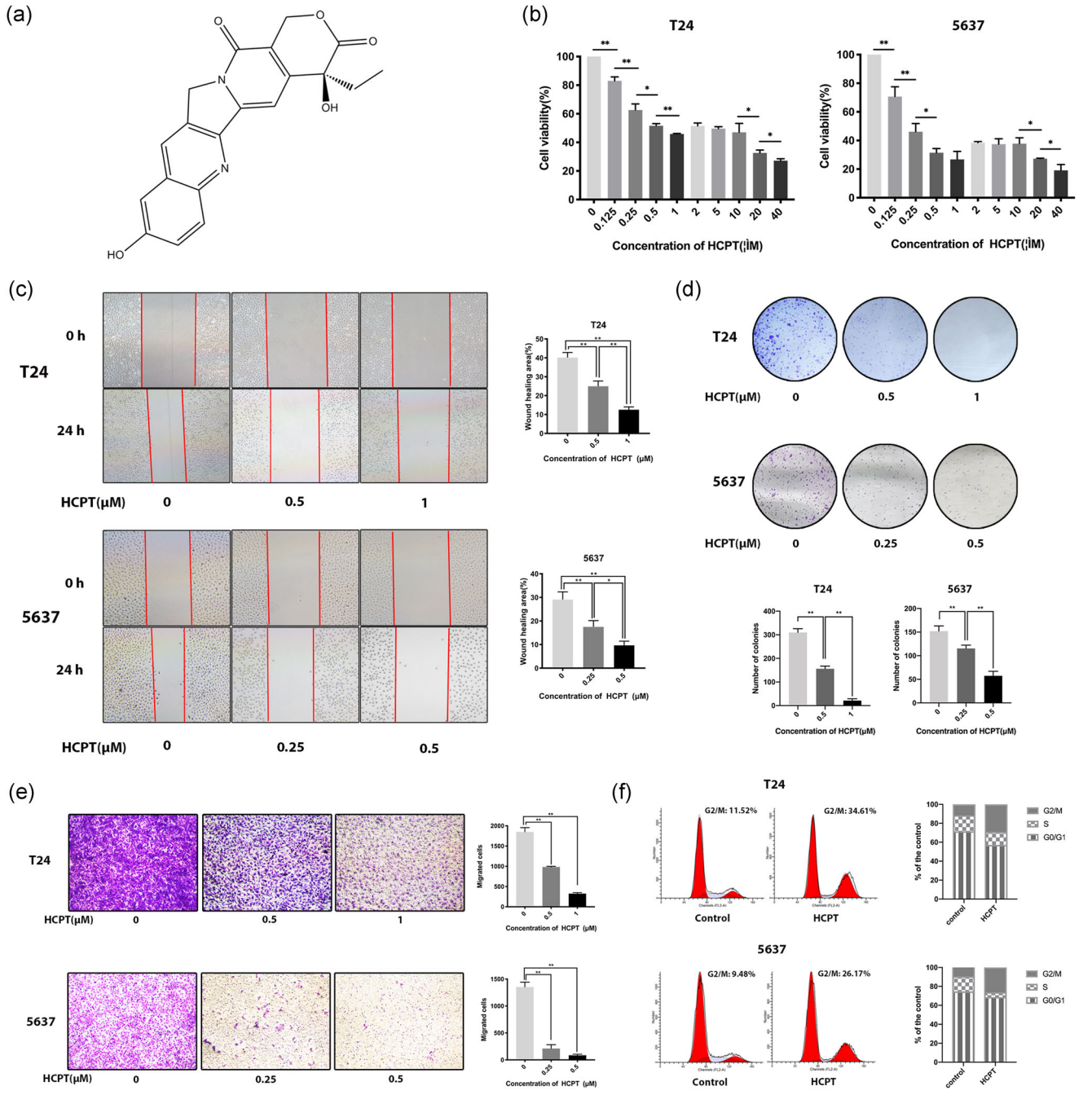

FIGURE 1 10-hydroxycamptothecin (HCPT) suppressed cell viability, proliferation, migration, and induced cell-cycle arrest of human bladder cancer cells. (a) The chemical structure of HCPT. Cells were treated with dimethyl sulfoxide (DMSO) or HCPT for 24 hr and (b) Cell viability of T24 and 5637 cells was determined by cell counting kit-8 (CCK-8) assay. (c) Wound healing assay of T24 and 5637 cells. (d) Colony-formation assay of T24 and 5637 cells. (e) Transwell assay of T24 and 5637 cells. (f) HCPT-induced G2/M phase arrest was analyzed by flow cytometry. The values were represented as mean ± standard deviation (SD) of three replicates. $^{*}p < .05$, $^{**}p < .01$

results, 0.5 and 1 μM HCPT were chosen as optimal concentrations to treat the two cell lines, respectively.

3.2 | HCPT-induced caspase-dependent apoptosis in human bladder cancer cells

Apoptosis is crucial in maintaining homeostasis as a programmed genetically process (Hagen et al., 2013; Ren et al., 2015; X. Zhang et al., 2015). Hence, whether or not HCPT induces apoptosis in bladder cells was further explored. Using Hoechst 33342 as the substrate, as shown in Figure 2a, we found that HCPT remarkably induced apoptosis represented as shrunken cells with abnormal nuclei (condensed or fragmented). Then, the expressions of apoptosis-related proteins were detected by western blot, and the results showed that HCPT activated Bax, cleaved caspsase-3 and cleaved PARP, while suppressing Bcl-2 level (Figure 2b). Next, the cells after

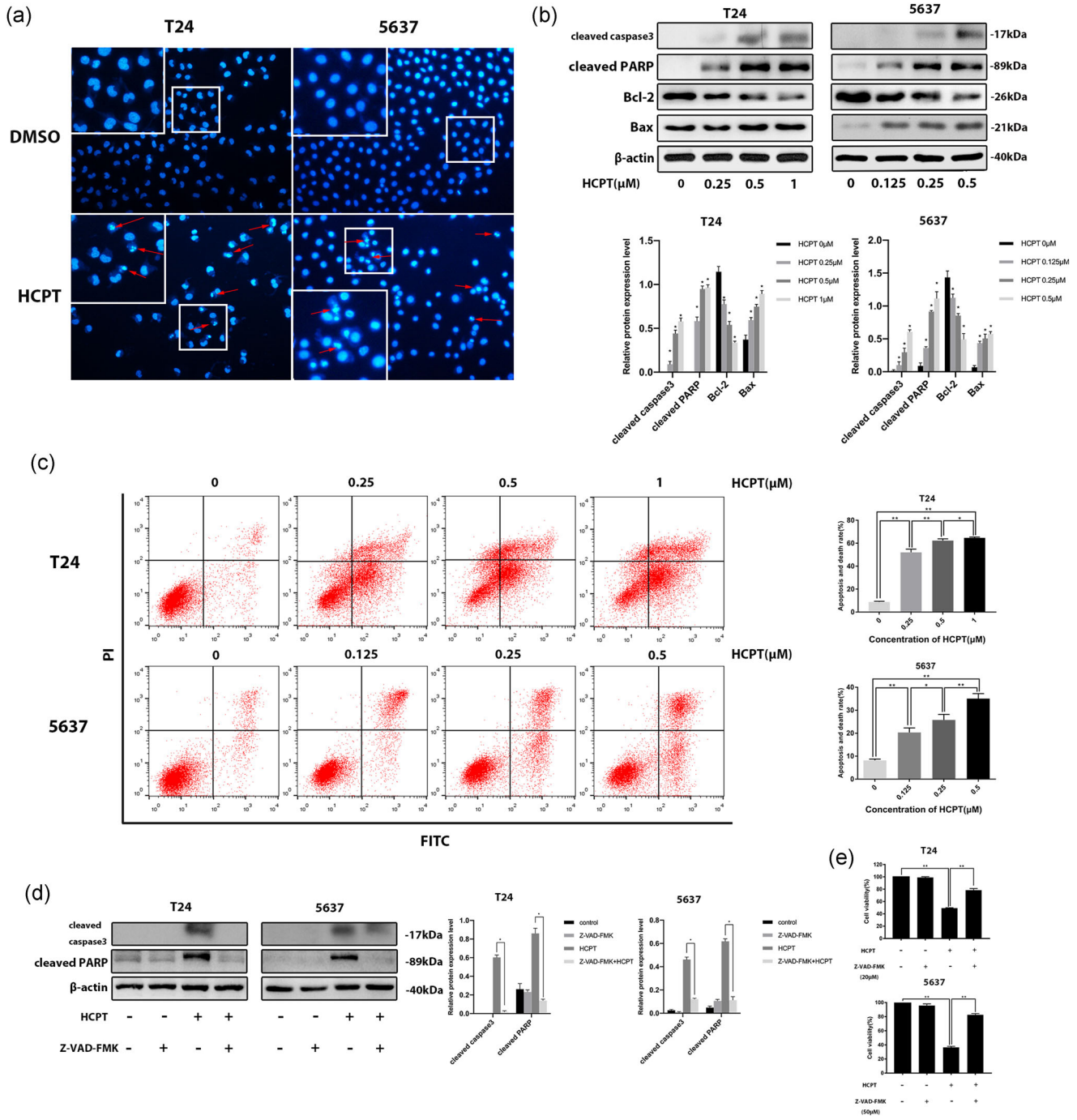

FIGURE 2 10-hydroxycamptothecin (HCPT) dose-dependently induced caspase-dependent apoptosis in human bladder cancer cells. (a) Representative images of apoptosis in T24 and 5637. The cells were treated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000), 1 and 0.5 μM HCPT for 24 hr, respectively. Cells were stained with Hoechst 33342. Abnormal and apoptotic cells are indicated by red arrows. (b) HCPT regulated the expression levels of apoptosis-related proteins in bladder cancer cells. Bax, Bcl-2, cleaved poly(ADP-ribose) polymerase (PARP), and cleaved caspase-3 levels were detected by western blot analysis. β-Actin was used as a loading control. (c) Cell apoptosis and death was measured by flow cytometry. The values were expressed as mean ± standard deviation (SD) of three independent experiments. (d) T24 and 5637 were treated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000), 1 and 0.5 μM HCPT with or without 20 and 50 μM Z-VAD-FMK for 24 hr, respectively. Caspase-related protein such as cleaved PARP and cleaved caspase-3 levels were detected by using western blot analysis. β-Actin was used as a loading control and (e) Cell counting kit-8 (CCK-8) assay was used to analyze the viability of T24 and 5637 cells. $^{*}p < .05$, $^{**}p < .01$

various concentrations of HCPT administration were stained with FITC and PI, and the apoptosis rates were detected by flow cytometer (Figure 2c,d). The results indicated that annexin V-positive cells (both early and late apoptosis) and dead cells were significantly increased after HCPT treatment dose-dependently.

Z-VAD(OMe)-FMK was used to inhibit the activity of caspase-3 and evaluate whether caspase-activation was necessary in HCPT-induced apoptosis. As shown in Figure 2e, Z-VAD(OMe)-FMK in combination with HCPT significantly decreased cleaved caspase-3 and cleaved PARP. Accordingly, we also observed the cell viability and apoptosis rate (Figures 2f and 7), and we found that Z-VAD(OMe)-FMK inhibited the caspase-dependent apoptosis induced by HCPT.

3.3 | HCPT stimulated autophagy in human bladder cancer cells

As there is no research concerning the relationship between HCPT and autophagy in bladder cancer, we transfected cells with lentivirus carrying mCherry-GFP-LC3B and then treated the cells with DMSO or HCPT for 24 hr. Once exposed to HCPT, LC3B aggregated on the membranes of autophagosomes represented as LC3B puncta (yellow fluorescence, R+G+). Autophagolysosomes displayed only red fluorescence (mCherry, R+G−) because the green fluorescent protein (GFP) fluorescence quenched under the acidic conditions once fusing with lysosome, which reflects the occurrence of autophagic flux. Differently, two fluorescences were diffused in the whole cell when incubated with DMSO. In this study, we regarded the totality of red puncta (R+G− and R+G+), which represents autophagosomes and autophagolysosomes, as the overall level of autophagy. Figure 3a showed that HCPT increased the totality of red puncta and red only puncta (R+G−) significantly. For further confirmation, we treated T24 and 5637 cell lines with 0.5 or 1 μM HCPT and observed the cellular autophagy under TEM. Autophagosomes were distinctly recognized with a double-membrane structure containing cellular components and high density of electrons. Accordingly, a dramatic increase of autophagosomes was observed in the HCPT-treated group (Figure 3b). LC3-II and SQSTM1/p62 were regarded as biomarkers of autophagy and autophagic flux in this study. (Hewitt et al., 2016). HCPT triggered significant increase in LC3B-II and with simultaneous decrease in p62 levels dose and time-dependently in two cell lines (Figure 3c,d). Furthermore, to distinguish if HCPT promoted the formation or inhibited the degradation of autophagosomes, we used bafilomycin A1 (BafA1) to additionally inhibit autophagosome clearance by lysosomes and to detect the autophagic flux (Yamamoto et al., 1998). The results showed that a significantly higher level of LC3B-II and less p62 were detected by western blot in BafA1 and HCPT co-treatment group as compared with the BafA1 group (Figure 3e). All together, these results strongly proved that HCPT could induce autophagy in T24 and 5637.

3.4 | HCPT triggered AMPK-mTOR-ULK1 pathway in human bladder cancer cells

AMPK, as a crucial energy sensor, regulates cellular energy metabolism when triggered by starvation or other adverse stimuli (Gallo et al., 2016; Ji et al., 2015; Sanduja et al., 2016). To determine whether AMPK was correlated with autophagy induced by HCPT and study the underlying molecular mechanism, the levels of p-AMPKα Thr172 and total AMPKα were determined by western blot analysis first. As the results showed in Figure 3c, we found obvious elevation of AMPKα phosphorylated at Thr172 in T24 and 5637 after HCPT treatment, whereas total AMPKα did not alter significantly. It indicated that HCPT might trigger the AMPK pathway, thus inducing autophagy. Previous studies reported that AMPK could enhance autophagy by inhibiting phosphorylated mTOR at Ser2448 and phosphorylating ULK1 at Ser555 (Li et al., 2018; Nwadike, Williamson, Gallagher, Guan, & Chan, 2018). According to this, we examined several autophagy-related downstream proteins of AMPK. Consistent with our preconception, HCPT did not have an impact on total mTOR and total ULK1, but significantly decreased p-mTOR and upregulated p-ULK1 in two cell lines (Figure 3c). These results demonstrated that HCPT could activate the AMPK-mTOR-ULK1 pathway and induce autophagy in human bladder cancer cells.

3.5 | Autophagy inhibition attenuated the HCPT-induced cytotoxic effect in human bladder cancer cells

To unmask the intricate interaction of autophagy and apoptosis, ATG7 siRNA and 3-methyladenine (3-MA) were used to block autophagy genetically and pharmacologically. As shown in Figures 4a and 5a, both siATG7 and 3-MA treatment significantly reduced the totality of autophagosomes and autophagolysosomes and the number of autophagolysosomes. Synergistically, blocking autophagy attenuated the cytotoxic effects on two bladder cancer cells (Figures 4b and 5b). Therefore, we hypothesized that the autophagy activated by HCPT might be a pro-apoptosis response. To verify this, we detected LC3B-II, ATG7, and apoptosis-related proteins by western blot analysis. Our results showed that siATG7 significantly decreased ATG7 expression. Meanwhile, siATG7 reduced LC3B-II expression as 3-MA did. We also observed the significant decrease of cleaved caspase-3 and cleaved PARP along with the elevated Bcl-2 (Figures 4c and 5c). Consistent with the above results, the subsequent cell apoptosis rate analysis confirmed that the suppression of autophagy obviously attenuated the apoptosis induced by HCPT (Figure 7). To sum up, our evidence indicated that autophagy contributed to HCPT-induced apoptosis in two cell lines.

3.6 | Enhanced autophagy aggravated HCPT-induced apoptosis in human bladder cancer cells

For further validation, we assessed the effect of rapamycin in cells treated with HCPT. As predicted, rapamycin dramatically increased the

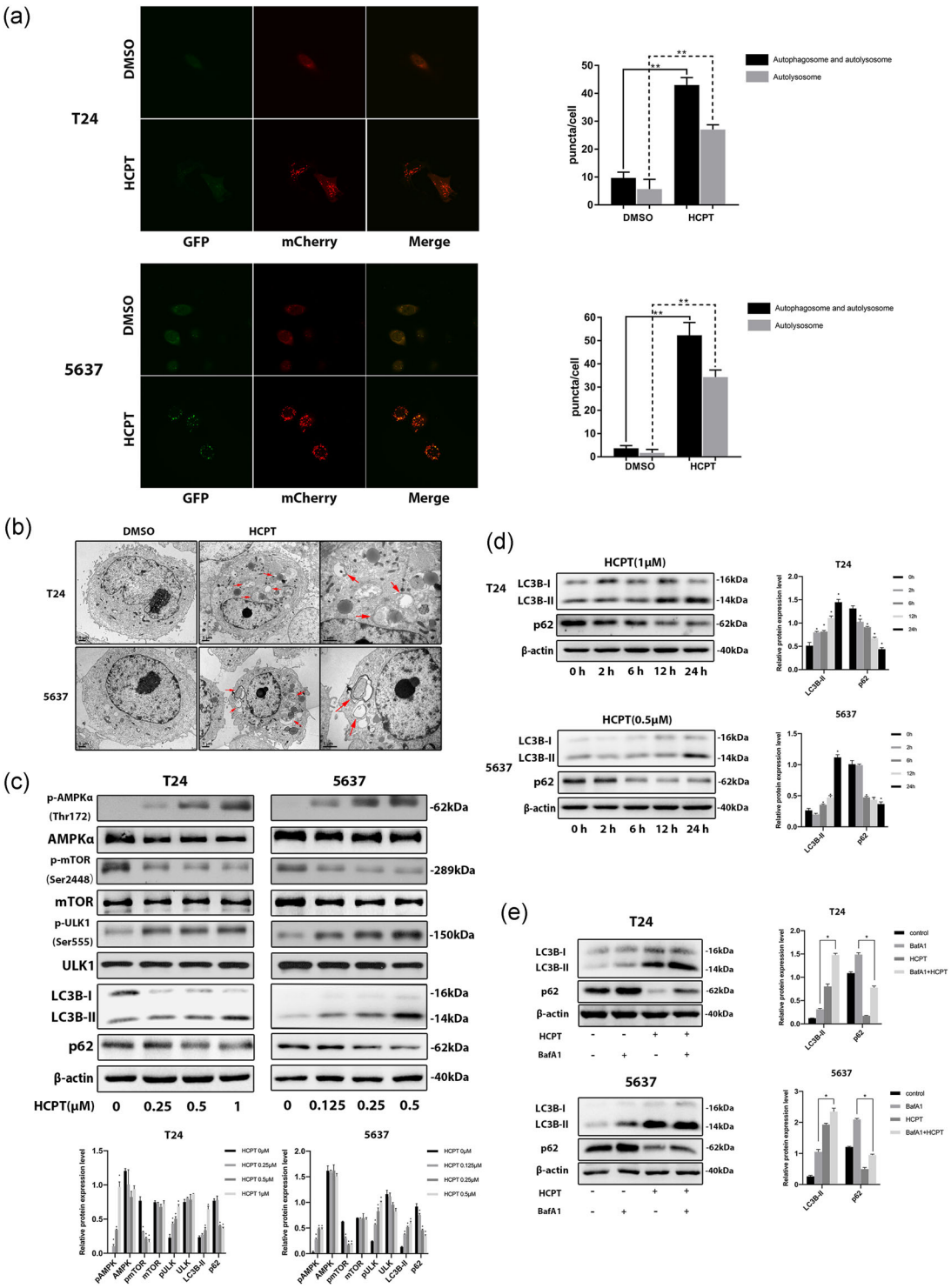

FIGURE 3 10-hydroxycamptothecin (HCPT)-induced autophagy through AMPK-mTOR-ULK1 axis in human bladder cancer cells. (a) T24 and 5637 pretransfected with lentivirus mCherry-GFP-LC3B were treated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for 24 hr, then observed under confocal fluorescence microscope. (b) The representative double-membrane structure of autophagic vesicles was shown in T24 and 5637 treated with HCPT (1 and 0.5 μM) for 24 hr using transmission electron microscopy (magnification, ×10,000 and ×20,000). The arrows indicate autophagic vesicles. (c) Cells were treated with various concentrations of HCPT for 24 hr and the expressions of AMPKα, p-AMPKα (Thr172), mTOR, p-mTOR (Ser2448), ULK-1, p-ULK1 (Ser555), LC3B-II, p62 were detected by using western blot analysis. (d) Cells were treated with various times of HCPT and the expressions of LC3B-II, p62 were detected by using western blot analysis. (e) T24 and 5637 were pretreated for 3 hr with 0.5 mM bafilomycin A1 (BafA1) followed by treatment with or without HCPT for 24 hr, the expressions of LC3B-II and p62 were determined by western blot. $^{*}p < .05$, $^{**}p < .01$

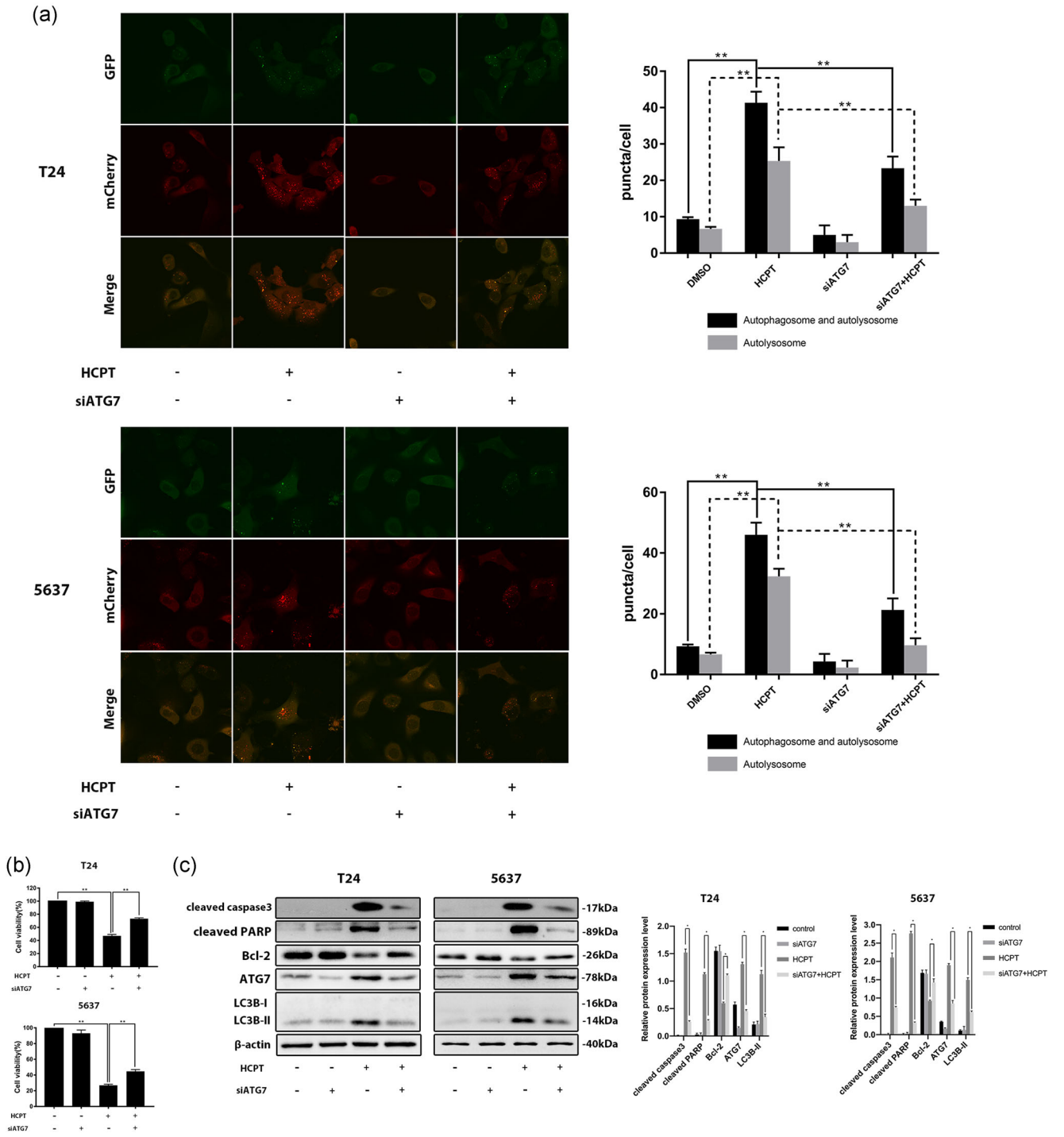

FIGURE 4 The impact of small interfering RNA (siRNA) ATG7 on 10-hydroxycamptothecin (HCPT)-induced anticancer effect. (a) T24 cells pretransfected with lentivirus mCherry-GFP-LC3B (10 MOI) for 18 hr were transfected with siRNA ATG7 or control siRNA for 48 hr, then incubated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. Cells were then imaged by confocal fluorescence microscopy. (b) Cell counting kit-8 (CCK-8) assay was used to analyze the cell viability. (c) Western blot analysis was used to analyze the protein level of ATG7, LC3B-II, cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, and Bcl-2. $^{*}p < .05$, $^{**}p < .01$

totality of autophagosomes and autophagolysosomes and the number of autophagolysosomes (Figure 5a), while it suppressed cell viability significantly (Figure 5d). Likewise, we found increased formation of LC3B-II, cleaved caspase-3, and cleaved PARP along with downregulated Bcl-2 (Figure 5e). Additionally, rapamycin also distinctly increased the apoptosis and dead cell rate when acting cooperatively with HCPT (Figure 7). The results above reconfirmed that autophagy played a cytotoxic role in HCPT treatment.

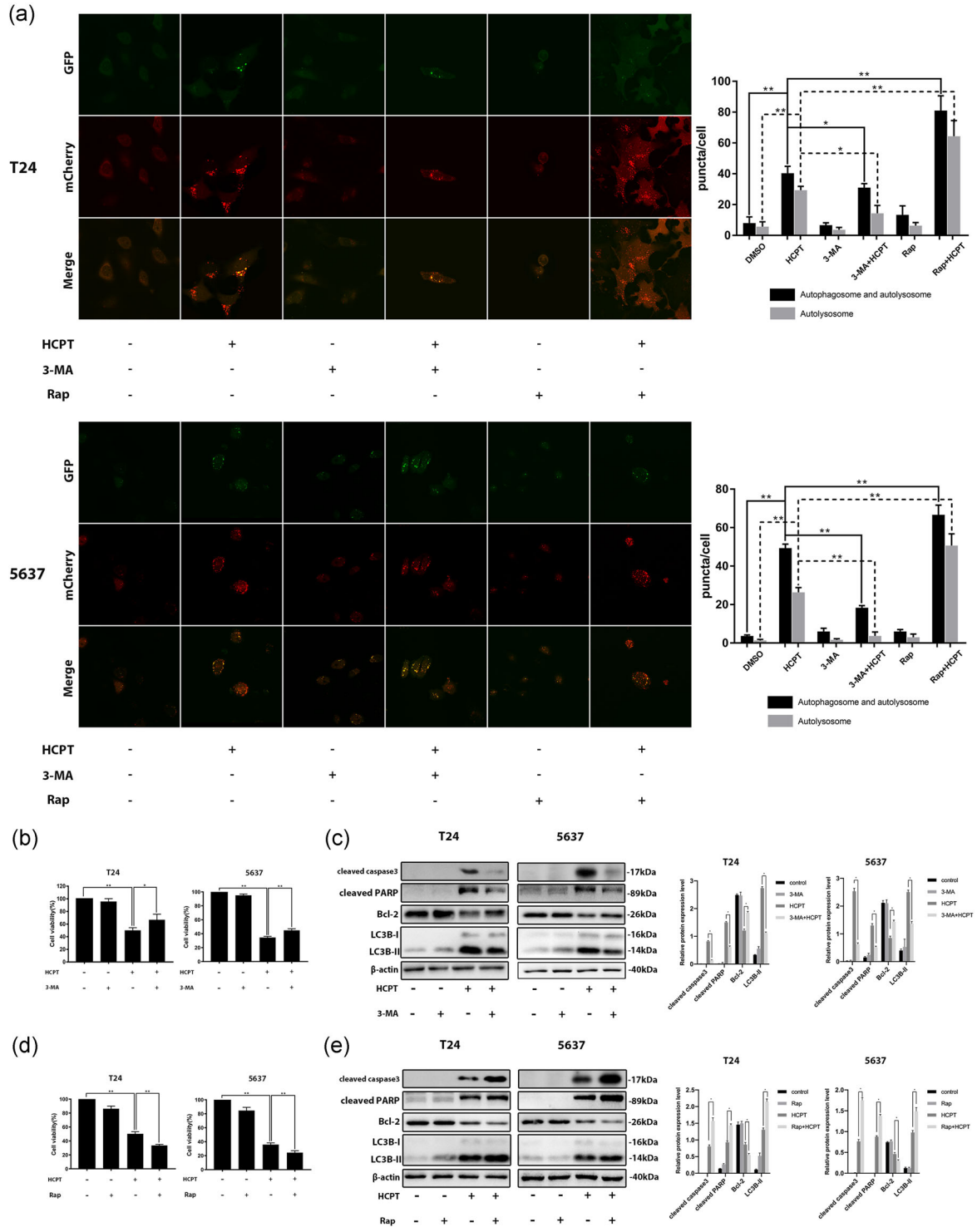

FIGURE 5 The impact of 3-methyladenine (3-MA) or rapamycin on 10-hydroxycamptothecin (HCPT)-induced an anticancer effect. (a) T24 and 5637 cells pretransfected with lentivirus mCherry-GFP-LC3B were pretreated with 3-MA (10 mM) or rapamycin (250 nM) for 4 hr, then incubated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. Cells were then imaged by confocal fluorescence microscopy. (b) T24 and 5637 cells pretreated with or without 3-MA (10 mM) for 4 hr were treated with DMSO (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. Cell counting kit-8 (CCK-8) assay was used to analyze the cell viability. (c) Western blot analysis was used to analyze the protein level of LC3B-II, cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3 and Bcl-2. (d) T24 and 5637 cells pretreated with or without rapamycin (250 nM) for 4 hr were treated with DMSO (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. CCK-8 assay was used to analyze the cell viability. (e) Western blot analysis was employed to analyze the protein level of LC3B-II, cleaved PARP, cleaved caspase-3 and Bcl-2. *$p < .05$, **$p < .01$

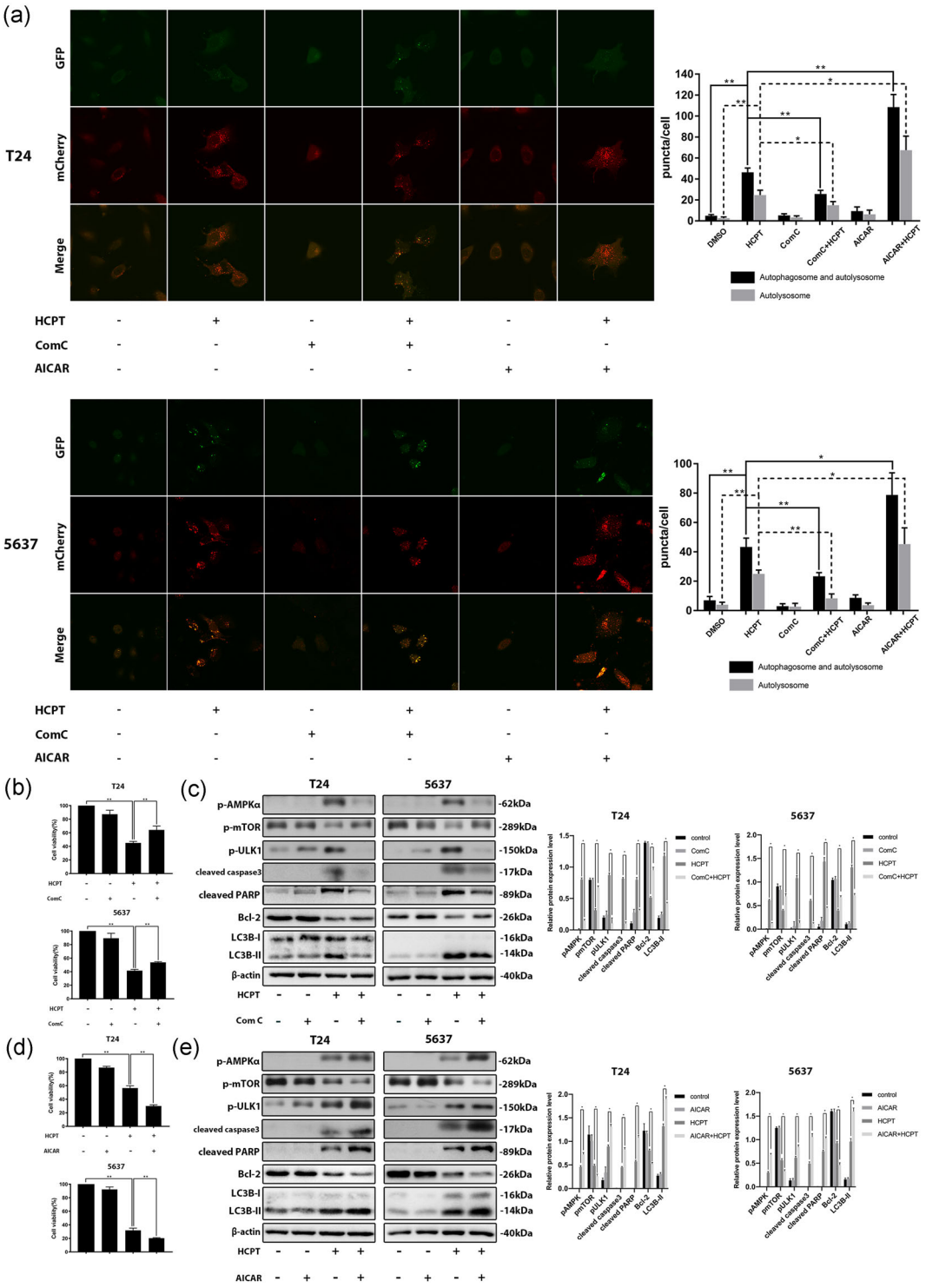

FIGURE 6 The impact of dorsomorphin or AICAR on 10-hydroxycamptothecin (HCPT)-induced anticancer effect. (a) T24 and 5637 cells pretransfected with lentivirus mCherry-GFP-LC3B were pretreated with dorsomorphin (10 μM) or AICAR (500 μM) for 4 hr, then incubated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. Cells were then imaged by confocal fluorescence microscopy. (b) T24 and 5637 cells pretreated with or without dorsomorphin (10 μM) for 4 hr were treated with DMSO (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. Cell counting kit-8 (CCK-8) assay was used to analyze the cell viability. (c) Western blot analysis was used to analyze the protein level of p-AMPKα (Thr172), p-mTOR (Ser2448), p-ULK1 (Ser555), LC3B-II, cleaved PARP, cleaved caspase-3 and Bcl-2. (d) T24 and 5637 cells pretreated with or without AICAR (500 μM) for 4 hr were treated with DMSO (v/v, 1:1,000) or HCPT (1 and 0.5 μM) for another 24 hr. CCK-8 assay was used to analyze the cell viability. (e) Western blot analysis was employed to analyze the protein level of p-AMPKα (Thr172), p-mTOR (Ser2448), p-ULK1 (Ser555), LC3B-II, cleaved PARP, cleaved caspase-3, and Bcl-2. *$p < .05$, **$p < .01$

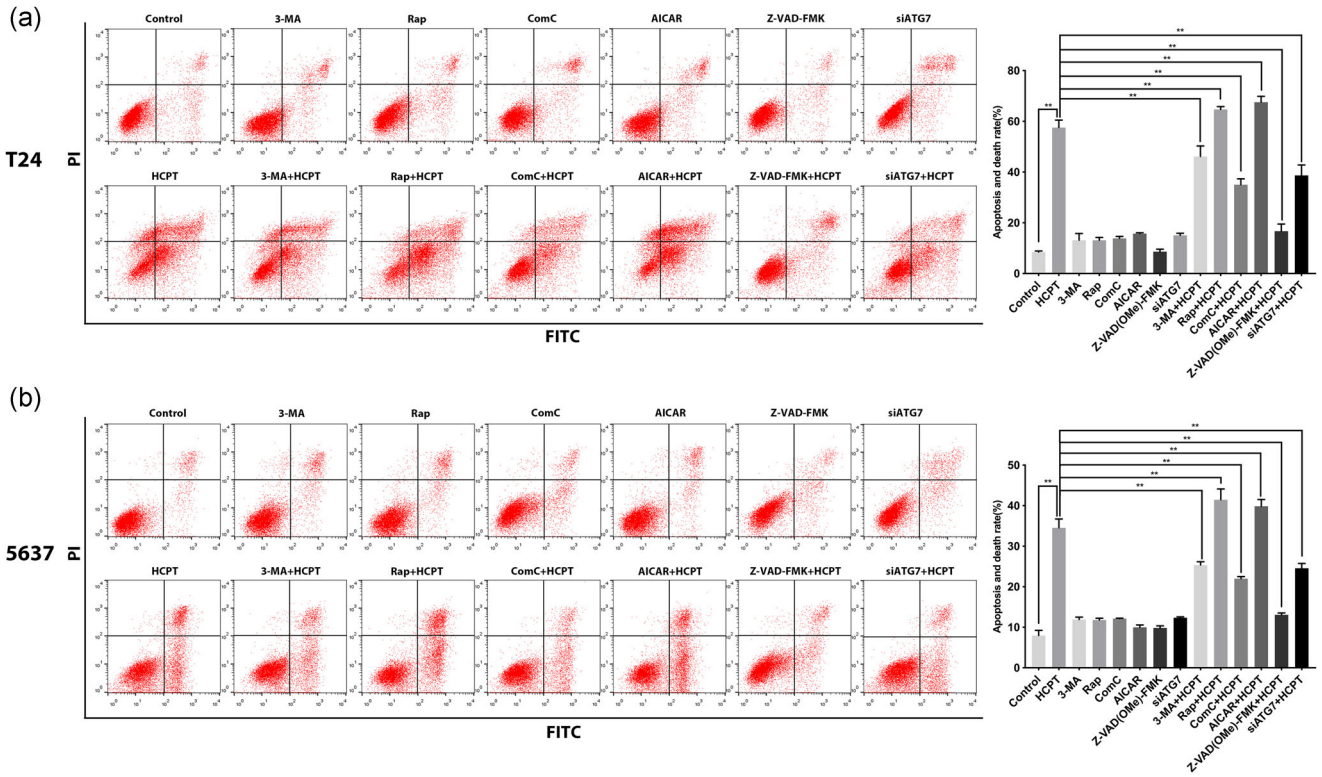

FIGURE 7 The impact of small interfering RNA (siRNA) ATG7, 3-methyladenine (3-MA), rapamycin, dorsomorphin, AICAR, or Z-VAD-FMK on HCPT-induced apoptosis. (a) T24 cells were transfected with siRNA ATG7 or control siRNA for 48 hr or pretreated with 3-MA (10 μM), rapamycin (250 nM), dorsomorphin (10 μM), or AICAR (500 μM) for 4 hr, then incubated with dimethyl sulfoxide (DMSO) (v/v, 1:1,000) or HCPT (1 μM) for another 24 hr. Z-VAD-FMK (20 μM) was co-treated with HCPT (1 μM) for 24 hr. Cell apoptosis and death were measured by flow cytometry. (b) 5637 cells were transfected with siRNA ATG7 or control siRNA for 48 hr or pretreated with 3-MA (10 μM), rapamycin (250 nM), dorsomorphin (10 μM), or AICAR (500 μM) for 4 hr, then incubated with DMSO (v/v,1:1,000) or HCPT (1 μM) for another 24 hr. Z-VAD-FMK (20 μM) was co-treated with HCPT (1 μM) for 24 hr. Cell apoptosis and death were measured by flow cytometry. $^{*}p < .05$, $^{**}p < .01$

3.7 | Activation of AMPK-mTOR-ULK1 by HCPT was critical in cytotoxic autophagy upon apoptosis in human bladder cells

To demonstrate that the AMPK-mTOR-ULK1 pathway participated in HCPT-induced autophagy and apoptosis, we employed dorsomorphin and AICAR to regulate the AMPK activity and evaluated the following effects. Dorsomorphin decreased the totality of autophagosomes and autophagolysosomes and the number of autophagolysosomes in T24 and 5637 after HCPT administration (Figure 6a). Meanwhile, it reversed HCPT-induced growth inhibition in these two cell lines (Figure 6b). Western blot analysis confirmed that dorsomorphin reduced the expressions of p-AMPKα, p-ULK1, and upregulated the level of p-mTOR conversely. At the same time, the formation of LC3B-II diminished dramatically along with decrease cleaved caspase-3 and cleaved PARP, and Bcl-2 were upregulated (Figure 6c). In contrast, activating AMPK by AICAR increased autophagosomes and autophagolysosomes (Figure 6a), AICAR further enhanced the cytotoxic effect of HCPT in T24 and 5637 cell lines (Figure 6d). Conversely, AICAR led to the higher expression of LC3B-II, p-AMPKα, and p-ULK1 along with the lower expression of p-mTOR. In accordance with previous results, AICAR promoted apoptosis represented as the increased cleaved PARP and cleaved caspase-3 and downregulated Bcl-2 level (Figure 6e). Finally, we examined the cell apoptosis and death by flow cytometry (Figure 7). The results further reconfirmed that AMPK-mediated autophagy contributed to HCPT-induced apoptosis.

4 | DISCUSSION

This study illustrated the anticancer effects of HCPT in human bladder cancer cells, and further exploration revealed that significant apoptosis and autophagy were induced simultaneously. Interestingly, HCPT-induced autophagy *per se* enhanced its apoptosis effect. These processes may interact partly through the AMPK-mTOR-ULK1 signaling pathway.

Bladder cancer is the ninth most common malignancy and also the second most common malignancy in the urinary system (Ferlay et al., 2015; Malats & Real, 2015; Miller et al., 2016). The high risk of recurrence and progression and long treatment cycle of post-operative bladder irrigation make it necessary and urgent to seek novel biological mechanisms and improve clinical strategy.

As medical and biological technologies advance remarkably, more natural plant extracts have been discovered to induce antitumor effects. Previous studies have reported that HCPT led to irreversible DNA damage via inhibiting DNA topoisomerase I and has the promising potential as a chemotherapy reagent (Urasaki et al., 2000; Wall & Wani, 1996; G. Zhang et al., 2011; R. Zhang et al., 1998). Nonetheless, the exact biological effects of HCPT, a natural alkaloid widely used in intravesical instillation, upon bladder cancer cells and underlying mechanism are still unclear.

Apoptosis is a programmed cell death process and represents as cell shrinkage, nuclear fragmentation, and chromatin condensation. Inducing apoptosis has been a very common biological mechanism of antitumor drugs. Bcl-2 and Bax are crucial apoptotic regulators and biomarkers (Zhou, Wang, & Zhu, 2016). Caspase-3, also named as CPP32, is an vital executor in the cell apoptosis process. PARP is known as one of the downstream components of caspase3 (J. Chen et al., 2004). We found that, upon HCPT administration, the protein level of Bcl-2 was decreased along with increased Bax. Meantime, HCPT-induced cleaved caspase-3 and PARP. Moreover, the combination of HCPT and Z-VAD(OMe)-FMK dramatically decreased cleaved caspase-3 and PARP and rescued the cells from cytotoxicity and apoptosis. Taken together, our results indicated that HCPT might potent anticancer activity by inducing caspase-dependent apoptosis in T24 and 5637 cell lines through the mitochondrial pathway.

In addition, previous studies have studied HCPT-induced autophagy and apoptosis in A549 cells, HeLa cells and human Tenon's capsule fibroblasts (Y. X. Cheng et al., 2016; Wei et al., 2018; Xu et al., 2014), but the intricate crosstalk between apoptosis and autophagy in human bladder cancer cells triggered by HCPT has not yet been confirmed. To verify autophagy, the switch of LC3-I to LC3-II and decreased SQSTM1/p62 are widely accepted biomarkers. Our findings showed that HCPT dramatically elevated expression of LC3B-II. Moreover, we transfected lentivirus containing mCherry-GFP-LC3B into bladder cancer cells and found that HCPT increased the autophagosomes and autophagolysosomes remarkably. The following Transmission Electron Microscopy reconfirmed the increased formation of autophagosomes in cells treated with HCPT. To ensure that HCPT triggered the formation of autophagosomes rather than inhibited the clearance of autophagosomes, we used BafA1 to determine autophagic flux. In summary, our results showed that HCPT triggered autophagy in human bladder cancer cells.

AMPK is vital in autophagy induction under stress, such as starvation (Vingtdeux et al., 2010). In our research, HCPT dramatically increased AMPKα phosphorylated at Thr172 and ULK1 phosphorylated at Ser555 but downregulated mTOR phosphorylated at Ser2448. Moreover, the co-treatment of HCPT and dorsomorphin or AICAR in the following detections showed consistent results. These findings confirmed that AMPK-mTOR-ULK1 pathway mediate the autophagy inducing after HCPT treatment in bladder cancer cells.

Once severe DNA damage occurred, various biological processes might be triggered by activating signaling pathways, including programmed cell apoptosis, AMPK/mTOR-dependent autophagy and cell-cycle arrest (Czarny, Pawlowska, Bialkowska-Warzecha, Kaarniranta, & Blasiak, 2015; Jorge et al., 2015; D. Zhang et al., 2015). On the basis of the previous studies and the targeting characteristic of HCPT, we supposed that HCPT might induce apoptosis and autophagy via DNA topoisomerase I inhibition and irreversible DNA damage.

Autophagy can maintain homeostasis and promote survival under stressful conditions; however, in some cases, it causes autophagic cell death and morbidity. We used siATG7, 3-MA, or AMPK inhibitor dorsomorphin to block the HCPT-induced autophagy genetically and pharmacologically, and the results showed that the inhibition of autophagy rescued cells from growth inhibition and apoptosis induced by HCPT in T24 and 5637, whereas rapamycin and AMPK activator AICAR enhanced the suppression in cell viability and apoptosis effect. These above results demonstrated that AMPK-mediated autophagy promoted apoptosis in HCPT administration.

In conclusion, our findings demonstrated that activation of AMPK-mTOR-ULK1 is crucial in autophagy induced by HCPT, which reinforces apoptosis and cytotoxicity upon HCPT treatment in T24 and 5637 cell lines. HCPT together with the autophagy activator would be a novel strategy for clinical treatment in bladder cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Study concept and design: Wang and Cao. Acquisition of data: Wang. Analysis of data: Wu, Huang, Zhang, and Hu. Technical or material support: Fan. Supervision: Jin. All authors have read and approved the final version of this manuscript and agree with the order.

DATA AVAILABILITY STATEMENT

Xiaodong Jin had full access to all the data in this study and takes the responsibility for the integrity of the data and the accuracy of the data analysis. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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