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Hydroxycamptothecin shows antitumor efficacy on HeLa cells via autophagy activation mediated apoptosis in cervical cancer

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Summary

Objectives: To investigate the effect and the mechanism of anti-tumor agent hydroxycamptothecin (HCPT) on HeLa cells in cervical cancer. *Materials and Methods:* Autophagy and apoptosis were detected by western blotting and the transfection of GFP-LC3 shRNA as well as Hoechst staining. *Results:* The authors found that the expression of the regulators of Beclin-1, p62, and microtubule-associated protein 1 light chain 3 (LC3) upregulated and then triggered the occurrence of cell autophagy. On the other hand, HCPT could induce to the formation of autophagy and resulted in cell apoptosis after autophagy. *Conclusion:* HCPT can alter cell autophagy and then trigger cell apoptosis to achieve antitumor effects.

Key words: HCPT; Cervical cancer; Autophagy; Apoptosis.

Introduction

Cervical cancer is one of the most common malignant tumors harmful to women's health. It is still the most common gynecological cancer worldwide with high morbidity and mortality, especially in developing countries [1,2]. Cervical squamous cell carcinoma is the most common pathological type [3]. Chemotherapy is a complementary comprehensive treatment strategy in addition to surgery and radiation therapy. However, the potential cellular and molecular mechanism of the drugs to the cervical cancer needs to be investigated in current research.

Autophagy is a kind of the eukaryotic cell apoptosis, also known as programmed cell death type II. Autophagy occurs at basal levels in most tissues and contributes to the routine turnover of cytoplasmic components which is involved in development, differentiation, and tissue homeostasis in various organisms as well as in the progress of certain human diseases [4, 5]. Multiple signals, including nutrient deprivation, endoplasmic reticulum stress, oxidative stress, and immune cell activation, stimulate autophagy [6]. Recent studies suggest that autophagy may be important in numerous diseases, including neurodegenerative disorders, bacterial and viral infections, cardiovascular disease, and the regulation of cancer development and progression [7]. The anticancer effect of autophagy has been renewed among oncologists which may represent an attractive therapeutic target of the anticancer agents [8, 9].

Hydroxycamptothecin (HCPT) is an anti-tumor agent by selective inhibitory effect on topoisomerase I [10, 11]. HCPT inhibits Top1 by stabilizing the covalent complex between Top1 and DNA, leading to enzyme-linked DNA strand breaks, also referred to as cleavage complexes, and it is less toxic in experimental animals and in human clinical evaluations [12, 13]. Therefore, HCPT is most likely to contribute to the treatment of malignant tumor including cervical cancer. However, the effect of HCPT on cervical cancer should be comprehensively investigated. The objective of this study was to determine the effect of HCPT on autophagy and apoptosis of HeLa cells in cervical cancer and to explore the molecular mechanism.

Materials and Methods

Cell viability

HeLa cells were seeded at 5,000 cells/well in 96-well plates and allowed to attach for 12 hours at 37°C under 5% CO_2 and ambient oxygen. Cells were treated with either HCPT at different concentrations (1, 2, 4, 6, 8 or 10 μM) or sterile saline which is a negative control and the authors determined the effects on cell viability using the CCK-8 assay. Each sample had six duplicate wells. Ten microliters of CCK-8 solution was then added to each well. After incubating for two hours, the absorbance at 450 nm was measured using a microplate reader. Results were expressed as a percentage of sample absorbance in relation to the control.

Western blot analysis

HeLa cells were plated onto 60-mm culture dishes with three-μM concentration HCPT for 12 and 24 hours. After washing in cold PBS, the harvested cells were lysed on ice for 30 minutes in 100 mmol/L lysis buffer. Extracts were clarified via centrifugation at 12,000 × r/min for 15 minutes. The supernatant was collected as

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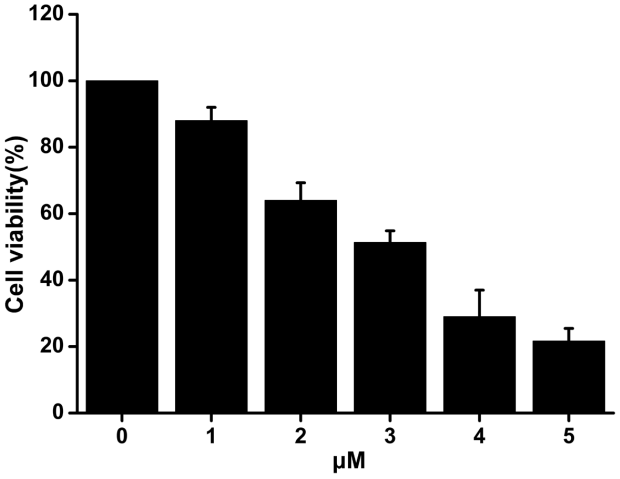

Figure 1. — The effect of HCPT on HeLa cells viability. HeLa cells were treated with the various concentrations of HCPT for 24 hours and analyzed for cell growth with CCK8 assay. The values were normalized to the results in untreated cells.

the total cellular protein extract. The protein concentrations were determined using a bicinchoninic acid protein assay kit. For western blot analysis, equal amounts of protein were run in each lane on a Tris-glycine gel using SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane. After blocking, the membranes were probed with the primary antibodies described above overnight at 4°C. After washing in TBST, the membrane was incubated with diluted horseradish peroxidase conjugated secondary antibodies at 37°C for one hour. After washing in TBST again, the proteins were visualized using an ECL detection kit and specific antibody-binding was visualized and quantified using Imaging J. Experiments were performed three times obtaining similar results.

Morphological changes during cell apoptosis

HeLa cells (10^6) were plated into six-well plates. After overnight incubation, cells were treated with HCPT in medium for 12 and 24 hours. The cells were washed in PBS three times and fixed with 4% paraformaldehyde for 15 minutes separately. Afterwards, the cells were stained with the Hoechst 33342 for ten minutes at 37°C in the dark. The cells were washed in PBS and morphological images were determined using a fluorescence microscope.

Cell autophagy rate

For transfection, HeLa cells were cultured in a six-well plate until they reached 60% - 70% confluence and then transfected with GFP-LC3 plasmid using lipofectamine 2000 in OPTIMEM according to the manufacturer's protocol. After six hours of transfection, the medium was removed and replaced with complete growth medium and samples were collected after 24 hours. Afterward, cells were treated with HCPT in medium for 12 and 24 hours. The cells were washed in PBS three times and fixed with 4% paraformaldehyde for 15 minutes separately.

Data analysis

All analyses were made using the SPSS 19.0 statistical software package. Data are presented as mean ± standard error of the mean (SEM) and depict the average of at least three independent

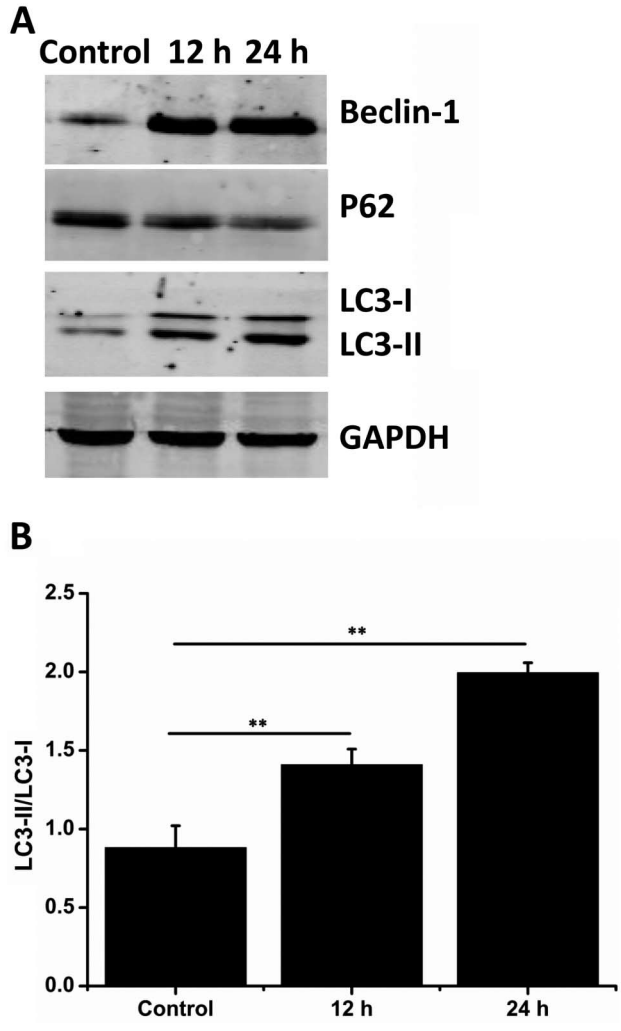

Figure 2. — HCPT induces the expression changes of proteins involved in autophagy in HeLa cells. HeLa cells were treated with HCPT at a dose of three μM for 12 and 24 hours. (A) Western blot analysis of the levels of Beclin-1, p62, and LC3. GAPDH was used as a loading control. (B) Densitometric analysis for the level of expression was preformed using Image J analysis. LC3-II/LC3-I ration. Error bar represents ± SEM. Statistical significance was determined by performing ANOVA. ** $p \leq 0.01$.

experiments. Morphological images are representative of at least three independent experiments with similar results. The variances in each group were compared using Levene's Test. All experiments were analyzed with a one-way analysis of variance (ANOVA) and statistical analyses were performed using LSD-t test and SNK-q test. A p value of less than 0.05 was considered to be significant.

Results

Effect of HCPT on HeLa cells viability

The authors assessed the effect of HCPT on Hela cells by measuring the cell viability with different drug doses

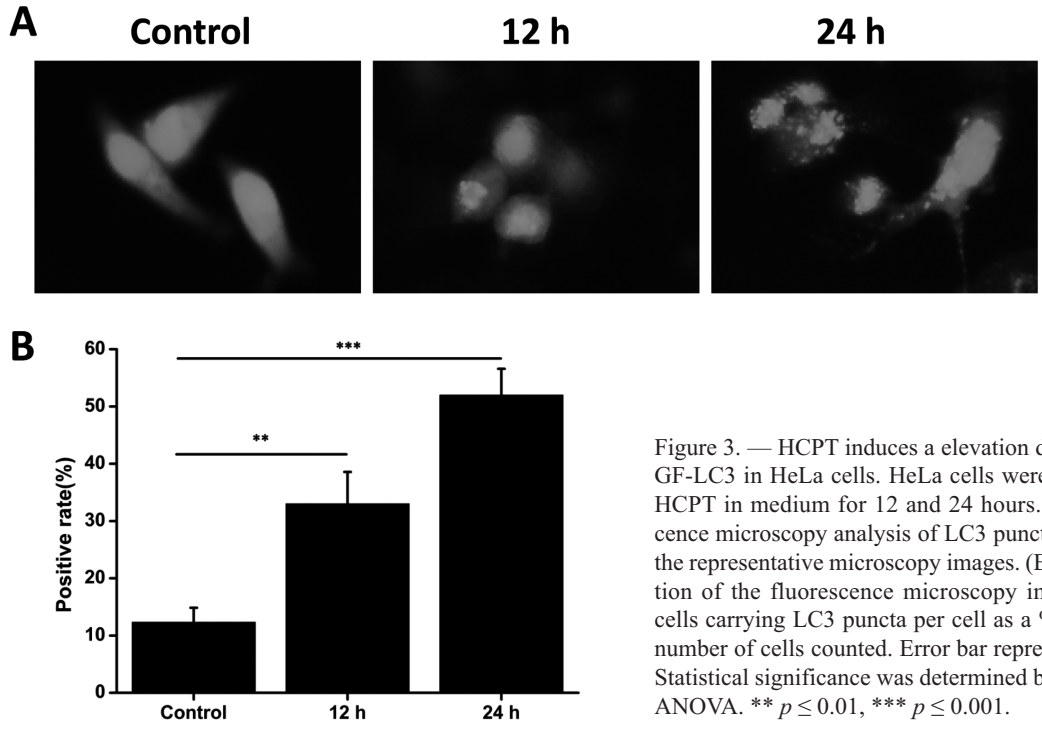

Figure 3. — HCPT induces a elevation distribution of GF-LC3 in HeLa cells. HeLa cells were treated with HCPT in medium for 12 and 24 hours. (A) Fluorescence microscopy analysis of LC3 puncta. Shown are the representative microscopy images. (B) Quantification of the fluorescence microscopy images for the cells carrying LC3 puncta per cell as a % of the total number of cells counted. Error bar represents ± SEM. Statistical significance was determined by performing ANOVA. ** $p \leq 0.01$, *** $p \leq 0.001$.

(Figure 1). HCPT significantly inhibited the growth of HeLa cells and decreased cell proliferation in HeLa cells in a dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) of HCPT was three μM and selected to explore the mechanism of HCPT in this study.

HCPT induces the expression changes of proteins involved in autophagy in Hela cells

To determine whether HCPT can alter autophagy, the authors treated Hela cells with HCPT at a dose of three μM for 12 and 24 hours. The conversion of LC3-I to LC3-II, a marker for autophagosome formation and the expression level of the p62 protein, which is degraded by autolysosomes and serves as a marker for autophagic flux, were determined by western blotting. Cells with HCPT maintained significantly higher levels of LC3-II and lower levels of p62 than the control group, and also in a time-dependent manner (Figure 2), suggesting that HCPT can lead to the upregulation of autophagy and by the time of exposure to HCPT, the autophagy enhanced.

HCPT induces an elevation distribution of GF-LC3 in HeLa cells

GFP-LC3 is considered as a specific marker for autophagosome formation. The HeLa cells were treated with HCPT for 12 and 24 hours after the transfection of GFP-LC3. Fluorescence microscopy was applied to count the total number of cells and the cells with positive autophagy point. Cells treated with HCPT maintained significantly higher levels of autophagosome with respect to control group (Figure 3), suggesting that HCPT can induce again the formation of autophagy.

HCPT induces morphology changes during HeLa cells' apoptosis

The authors treated HeLa cells with HCPT at a dose of three μM for 12 and 24 hours and fixed with 4% paraformaldehyde. Then they stained the cells with the Hoechst 33342. As shown in Figure 4, in contrast to control cells, fluorescence microscopic results showed that the nuclei of HeLa cells treated with HCPT for 24 hours were shrunken and hyperchromatic, but the cell nucleus treated for 12 hours had no significant changes, suggesting the cell apoptosis appear after autophagy.

HCPT induces the expression changes of proteins involved in apoptosis in HeLa cells

To investigate the cell apoptosis induced by HCPT, the proteins involved in apoptosis was assessed by western blotting (Figure 5). The proteins of Bax and cleave-caspase3 that play a major role in the apoptotic process showed an elevation, while the expression of inhibitor of apoptosis protein Bcl-2 downregulated in the cells treated with HCPT for 24 hours and in the cells treated for 12 hours the expression of these proteins did not have obvious changes, suggesting that cell apoptosis appeared again after autophagy.

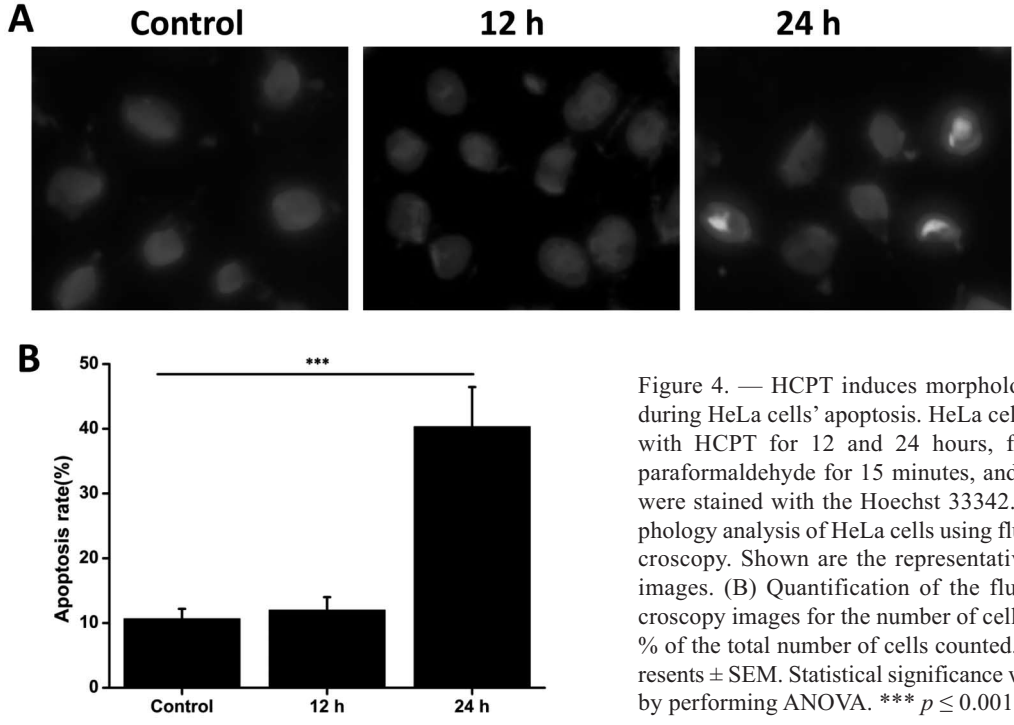

Figure 4. — HCPT induces morphological changes during HeLa cells' apoptosis. HeLa cells were treated with HCPT for 12 and 24 hours, fixed with 4% paraformaldehyde for 15 minutes, and then the cells were stained with the Hoechst 33342. (A) The morphology analysis of HeLa cells using fluorescence microscopy. Shown are the representative microscopy images. (B) Quantification of the fluorescence microscopy images for the number of cell apoptosis as a % of the total number of cells counted. Error bar represents ± SEM. Statistical significance was determined by performing ANOVA. *** $p \leq 0.001$.

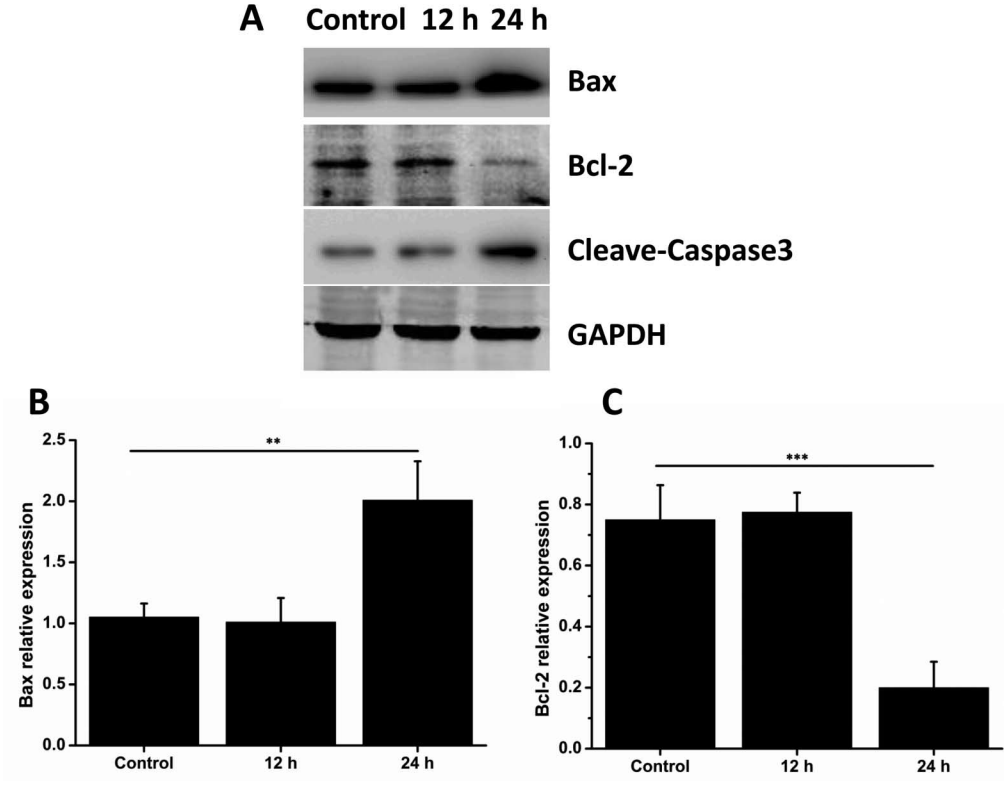

Figure 5. — HCPT induces the expression changes of proteins involved in apoptosis in HeLa cells. HeLa cells were treated with HCPT at a dose of three μM for 12 and 24 hours. (A) Western blot analysis of the levels of Bax, Bcl-2 and cleave-Caspase3. GAPDH was used as a loading control. (B and C) the expression of Bcl-2 and Bax was determined using western blotting. The staining intensity of the bands from all images was densitometrically quantified and normalized to GAPDH. Error bar represents ± SEM. Statistical significance was determined by performing ANOVA. ** $p \leq 0.01$, *** $p \leq 0.001$.

Discussion

HCPT, a pentacyclic alkaloid isolated from the Chinese tree Camptotheca acuminata, is reported to have a potent antitumor activity by its selective inhibitory effect on topoisomerase I and has been widely used [14]. HCPT has more stability and less adverse reactions and also induces the apoptosis of a variety of tumor cells and part of non-neoplastic cells. Cervical cancer is the second common gynecologic malignant tumor in the world killing more than ten thousands of persons every year and the incidence of it increasingly affects younger subjects. The main therapeutic intervention is still the comprehensive treatment of radiotherapy and chemotherapy [1].

In this study, the authors first demonstrated that HCPT can induce the autophagy of HeLa cells in a time-dependent manner by western blotting and the transfection of GFP-LC3 shRNA. LC3 is localized to isolated membranes and playing crucial roles in the formation of autophagosomes, therefore is now widely used as a key molecule to monitor autophagosome formation [15, 16]. The elevated expression of Beclin-1, which is the regulator of autophagy in mammals can induce the formation of autophagy [17, 18]. With the effect of HCPT, these two proteins of LC3 and Beclin-1, the markers for autophagy have a significant upregulation. Evidence suggests that in the development of cervical cancer, the expression of proteins related autophagy was suppressed and then downregulated the formation of autophagy, therefore causing the growth of tumor [19-21]. In addition, some studies have found that the growth of cervical cancer cells were significantly inhibited with the overexpression of proteins related autophagy [22, 23]. Therefore, inducing the formation of autophagy has a significant potential to be one of strategies for the treatment of cervical tumor in the future, and HCPT achieves the goal of antitumor by inducing the formation of autophagy.

In addition to upregulating the level of autophagy, the present authors also confirmed that HCPT can lead to cell apoptosis by the results of western blot and Hoechst staining, and apoptosis appeared after the appearance of autophagy, suggesting that HCPT elevated the level of autophagy firstly and then triggered the appearance of apoptosis, eventually leading to the death of cells and achieving the goal of antitumor.

Many studies have demonstrated an extensive crosstalk between autophagy and apoptosis and there are some similarities between them. Some arguments are that autophagy is necessary for the development of apoptosis, and the inhibition of autophagy will delay the occurrence of apoptosis, while the elevation of autophagy leads to the activation of apoptosis pathway. In this study, the authors investigated that in cervical cancer HCPT achieved the goal of antitumor by upregulating autophagy and then triggering the development of apoptosis, suggesting a complex interrelationship between autophagy and apoptosis [24-26]. The autophagosomes were increased and the expression of Beclin-1 and LC3 was significantly increased in HeLa cells treated with cisplatin and endoplasmic reticulum stress pathway was activated, suggesting that the role of endoplasmic reticulum stress-autophagic response in cisplatin-induced cervical cancer HeLa cell death [27]. A large amount of research still needs to reveal the relationship between autophagy and apoptosis.

In summary, the present authors first provided the evidence regarding the mechanism of the antitumor effect of HCPT. HCPT can alter cell autophagy and then trigger cell apoptosis to achieve antitumor effects. These findings indicate a new regulatory mechanism of HCPT that will be helpful in the development of more effective therapies in treating cervical cancer.

Acknowledgments

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