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Carfilzomib Induces Apoptosis of Endometrial Carcinoma Cells by Activating Endoplasmic Reticulum Stress

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Abstract

Objective: Carfilzomib induces endometrial carcinoma cell apoptosis by activating the endoplasmic reticulum stress signaling pathway.

Methods: Two endometrial cancer cell lines, HEC-1-A and Ishikawa, were treated with Carfilzomib. Flow cytometry was used to detect cancer cell apoptosis at different concentrations and times. The expression of *GRP78* and *CHOP* genes in endoplasmic reticulum stress was detected by qRT-PCR. Western Blot method was used to examine the effect of Carfilzomib on the expression of apoptosis-promoting factors (Bim, Puma, Noxa, and Bax) and endoplasmic reticulum stress-related factors (*GRP78* and *CHOP*) in endometrial cancer cells.

Results: Flow cytometry showed that Carfilzomib time- and dose-dependently induced the apoptosis of two endometrial cancer cell lines. qRT-PCR showed that Carfilzomib significantly upregulated *GRP78* and *CHOP* expression. Western blot showed that the apoptosis-promoting factors Bim, Puma and Noxa were upregulated at the protein level, while changes in *GRP78* and *CHOP* were dose-dependent.

Conclusion: The effect of Carfilzomib on endometrial cancer cells shows that the drug causes tumor cell apoptosis through the activation of the endoplasmic reticulum stress signal pathway in cancer cells.

Introduction

The ubiquitin-proteasome pathway plays a pivotal role in cell apoptosis, providing a new direction for the search for tumor therapy. Although the mechanism of proteasome inhibitors promoting tumor cell apoptosis is still unclear, previous studies suggest that it is closely related to endoplasmic reticulum stress [1]. Therefore, clarifying the signal pathway of proteasome inhibitors activating tumor cell apoptosis provides a theoretical basis for treating malignant tumors with proteasome inhibitors.

The Endoplasmic Reticulum (ER) is the center of protein synthesis and transport, which plays a role in maintaining a stable cell microenvironment (ER homeostasis). The proteasome is responsible for protein degradation in cells. The breakdown or destruction of the proteasome can offset the homeostasis of ER, leading to the accumulation of misfolded and unfolded proteins [2].

Proteasome Inhibitors (PIs) block the action of the proteasome by selectively and irreversibly binding to chymotrypsin-like sites in the protein hydrolysis core. Because PIs can disrupt the process of cell cycle regulation, they are used to induce programmed cell death and reduce the viability of cancer cells through classical and/or ER stress pathways [3]. Previous studies revealed the function of PIs in multiple myeloma (a malignant blood tumor) and adrenal cortical carcinoma (a solid tumor), revealing the differentiation behavior of tumor cells under ER stress induced by PIs [4,5].

Carfilzomib is a second-generation proteasome inhibitor approved worldwide after Bortezomib, which was the first multiple myeloma treatment drug approved by the US FDA in recent years [4]. Previous studies of our research group showed that Carfilzomib was effective on several tumor cell lines. MTT test showed that the drug had the highest growth inhibition rate on two endometrial cancer cells, HEC-1-A and Ishikawa. Therefore, HEC-1-A and Ishikawa were used for our current study. Previously published papers clarified the effect of Carfilzomib on the expression of cell cycle-related proteins to inhibit cell proliferation [6,7].

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In this report, we investigated the molecular role of Carfilzomib on endometrial cancer cells and whether it caused tumor cell apoptosis by activating the ER stress signaling pathway in cancer cells.

Materials, Methods

Drugs and antibodies

Carfilzomib, purchased from LC Laboratories (Woburn, MA, USA), was reconstituted in Dimethyl Sulfoxide (DMSO) at a stock concentration of 10 mmol/L and stored at – 20°C. The stock was diluted in DMSO to the needed concentrations (0 nM [no Carfilzomib, only DMSO], 12.5 nM, 25 nM, 50 nM, 100 nM). The final concentration of DMSO was <0.01% in the cell culture medium. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich; Schnelldorf, Germany) supplemented with 10% (w/v) heat-inactivated fetal bovine serum (FBS; HyClone; Logan, UT, USA), 100 U/mL penicillin, and 50 g/mL streptomycin at 37°C in 5% CO_2.

Primary antibodies (rabbit) used in this study included: Anti-Bcl2, anti-Mcl-1, anti-Bcl-x1, anti-Bax, anti-Bim, anti-Puma, anti-β-actin, anti-Noxa, anti-*GRP78* (Glucose-Regulated Protein 78), and anti-*CHOP* (C/EBP-Homologous Protein). All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary HRP-conjugated goat anti-rabbit antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell culture

The HEC-1-A (moderately-differentiated endometrioid, more sensitive to Carfilzomib) and Ishikawa cell (well-differentiated endometrioid, less sensitive to Carfilzomib) lines were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Sigma-Aldrich; Schnelldorf, Germany) supplemented with 10% (w/v) heat-inactivated FBS (HyClone; Logan, UT, USA), 100 U/mL penicillin, and 50 g/mL streptomycin at 37°C in 5% CO_2.

Flow cytometry

Apoptotic cells were evaluated using the annexin V-FITC apoptosis detection kit (BD Biosciences, NJ, USA). All experimental steps were performed in accordance with the protocols of the manufacturer. Briefly, 1 × 10^6 cells were washed twice with Phosphate-Buffered Saline (PBS) and stained with 100 µL of binding buffer containing 5 µL of 7-amino-actinomycin D and 5 µL of annexin V-FITC. Cells were incubated at 4°C for 15 min, resuspended in 400 µL of binding buffer, and immediately analyzed using a Fluorescence-Activated Cell Sorting (FACS) flow cytometer (BD Biosciences); 10,000 events were recorded and analyzed.

Western blotting

All experimental steps were performed in accordance with the protocols of the manufacturer. Briefly, cells were lysed with Radio Immunoprecipitation Assay (RIPA) buffer (Roche Applied Science; Mannheim, Germany) containing a protease inhibitor cocktail and phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bicinchoninic Acid (BCA) protein assay kit (Pierce; Rockford, IL, USA). Equal amounts of proteins were separated using 12% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electrophoretically transferred to a Polyvinylidene Fluoride (PVDF) membrane (Millipore Corporation; Bedford, MA, USA). The membranes were blocked for 1 h at room temperature with 5% nonfat milk. Next, the membranes were incubated with primary antibodies against Bax, Mcl-1, Bcl-2, Bcl-x1, Bim, Puma, Noxa, *GRP78*, and *CHOP* overnight at 4°C. The membranes were washed four times and incubated with anti-rabbit IgG (1:1,000) conjugated to Horseradish Peroxidase (HRP) at 37°C for 1 h. Blots were visualized using Enhanced Chemiluminescence (ECL) reagents (Millipore Corporation; Bedford, MA, USA).

Quantitative RT-PCR

All experimental steps were performed in accordance with the manufacturer's instructions. Briefly, RNA extraction was performed using the RN easy kit (QIAGEN, Dusseldorf, Germany), and the isolated Total RNA was reverse-transcribed by the Verso cDNA synthesis kit (Thermo Scientific, MA, USA). qRT-PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) on a 7900 HT Fast Real-Time PCR System from Thermo Fisher Scientific (Waltham, MA, USA) with gene-specific primers (Table 1). The cycling conditions were as follows: Pre-denaturation at 95°C for 2 min, 40 cycles of 95°C for 1 min, 60°C for 30 sec, and 72°C for 1 min, then extension at 72°C for 7 min. Cycle threshold (Ct) values were collected for β-actin and the genes of interest during the log phase of the cycle. Quantification of target gene expression was normalized against β-actin using the Ct method.

Cell viability assays

After standard cell cultivation for 24 h, the HEC-1-A and Ishikawa cell lines were seeded in a 96-well plate (8 × 10^3 cells per well) with graded doses of medicine for a further 24 h and subjected to standard MTT analysis. Briefly, 200 µl of solution (containing 0.5 mg/mL of MTT; Omega Biotech, Norcross, GA, USA) was added at the indicated time points at 37°C. After cell culture for 4 h, cells in each well were lysed in 150 µl of DMSO for 15 min at room temperature. The inhibitory rate (%) was evaluated by an FLUO star OPTIMA enzyme-labeled instrument from the (BMG Labtech, Offenburg, Germany) at OD_{490}.

Statistical analysis

Data are presented as mean ± Standard Error (SE). A one-way Analysis of Variance (ANOVA) was used to compare the means of the different testing groups and to calculate the p-value. Significance was set at $p<0.05$. The Coefficient Indices (CIs) of Carfilzomib (nM) and UMI-77 (µM) were calculated using the computer program CompuSyn (18) by entering the inhibitory rates of the different groups.

Results

Flow cytometry

Flow cytometry Annexin V-FITC and PI staining method was used to detect the effect of different Carfilzomib concentrations on HEC-1-A and Ishikawa cell apoptosis after 24 h (Figure 1). The flow cytometry results showed that the apoptosis rate of the two cell lines increased significantly with the increase of Carfilzomib concentration compared with the control group.

After 48 h of Carfilzomib treatment (Figure 2), flow cytometry results showed that, compared with the control group, the apoptosis rate of the two cell lines increased significantly with the increase of drug concentration and exceeded the apoptosis rate of the corresponding Carfilzomib concentration for 24 h.

Western blot

Western blot was used to detect the effect of Carfilzomib on Bim, Puma, and Noxa proteins. Notably, these proteins are the BH3-only members of the Bcl-2 family of apoptosis-promoting proteins.

Table 1: qRT-PCR primer.

Gene	Forward sequences (5'-3')	Reverse sequences (5'-3')
β-actin	ATCGTGCGTGACATTAAGGAGAAG	AGGAAGGAAGGCTGGAAGAGTG
GRP78	AGGAGGAGGACAAGAAGGAGGAC	CAGGAGTGAAGGCGACATAGGAC
CHOP	TGCTTCTCTGGCTTGGCTGAC	CCGTTTCCTGGTTCTCCCTTGG

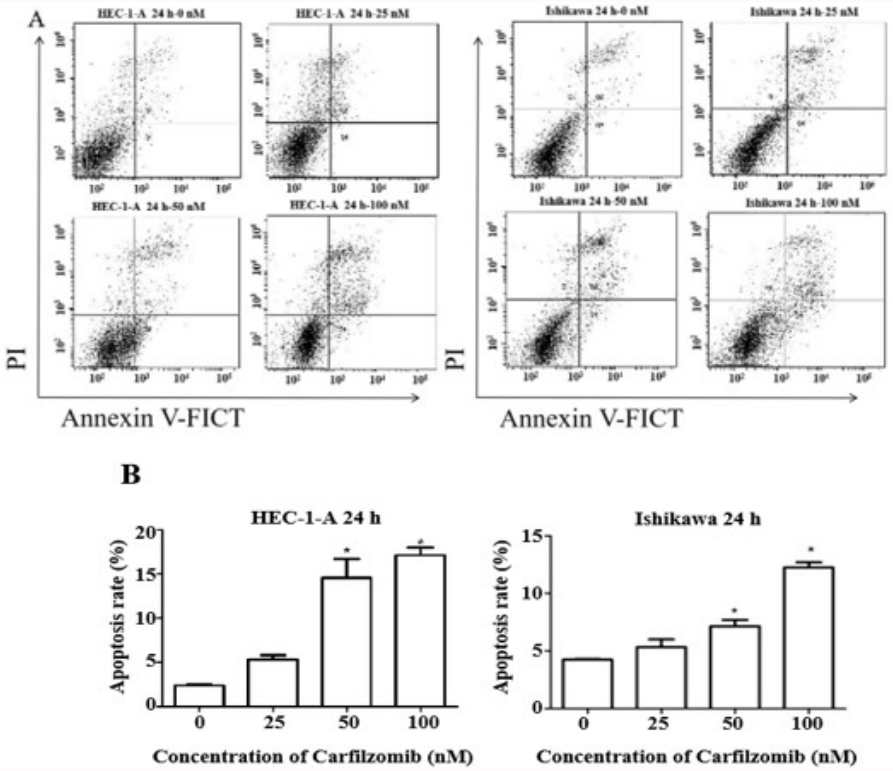

Figure 1: Effect of Carfilzomib on HEC-1-A and Ishikawa cell apoptosis after 24 h (A) After being exposed to Carfilzomib for 24 h, Annexin V/PI staining was used to detect HEC-1-A and Ishikawa cell apoptosis by flow cytometry. (B) Statistical diagram of apoptosis rate of HEC-1-A and Ishikawa cells (n=3) compared with the control group; *p<0.05, **p<0.01.

Examining the effect of Carfilzomib on apoptosis-promoting proteins revealed that Carfilzomib significantly increased the expression of Bim, Puma, and Noxa proteins, the BH3-only members of apoptosis-promoting protein family; however, Carfilzomib exerted no significant effect on the expression of Bax (Figure 3).

To verify that Carfilzomib causes ER stress in endometrial cancer cells, we used Western blot to detect the effects of Carfilzomib on *GRP78* and *CHOP* in two endometrial cancer cell lines. The results showed that Carfilzomib could significantly upregulate *GRP78* and *CHOP* protein expression (Figure 4).

Real-time PCR

To detect the changes of *GRP78* and *CHOP* at the transcription level, endometrial cancer cells were treated with Carfilzomib for different length of time, and RNA was extracted from the cells. The downstream products of reverse transcription were used for a real-time PCR experiment, which showed that Carfilzomib could significantly induce the transcription of *GRP78* and *CHOP* compared with the control group (Figure 5).

Discussion

Carfilzomib, a proteasome inhibitor, inhibits the proteasome activity in cancer cells, resulting in large intracellular protein depositions. The high *GRP78* protein expression supports that Carfilzomib causes ER stress in endometrial cancer cells [8]. According to previous studies, under continuous and severe ER stress, dimerization occurs after the dissociation of PERK and *GRP78*, upregulating PERK phosphorylation and p-PERK-specific phosphorylation of eIF2 α. When ER stress is too severe, a large amount of p-eIF2 α enables ribosomes to reach the open reading frame of ATF4 and allows ATF4 transcription. After ATF4 enters the nucleus, *CHOP* transcription can be activated, triggering the *CHOP* pathway to induce cell apoptosis. After unfolded protein response activation, ATF6 is transported to the Golgi apparatus and split in to 50 kDa p50ATF6. IRE1 α has endonuclease activity after being activated by self-phosphorylation. In addition, activated IRE1 α can specifically excise a 26-base intron from *XBP1* mRNA, changing the open reading frame of *XBP1* mRNA. Its translation product *XBP1* can promote the expression of unfolded protein reaction target molecules containing ERS response Elements (ERSE), such as *GRP78* and GRP94, to reduce or terminate the endoplasmic reticulum stress response, resetting cellular homeostasis [9-12].

Under normal circumstances, *GRP78* acts as a molecular chaperone and binds to the ER transmembrane protein (*GRP78*/BIP) to inhibit the polymerization of ER transmembrane protein; maintaining an inactive state as a whole. When ER stress occurs,

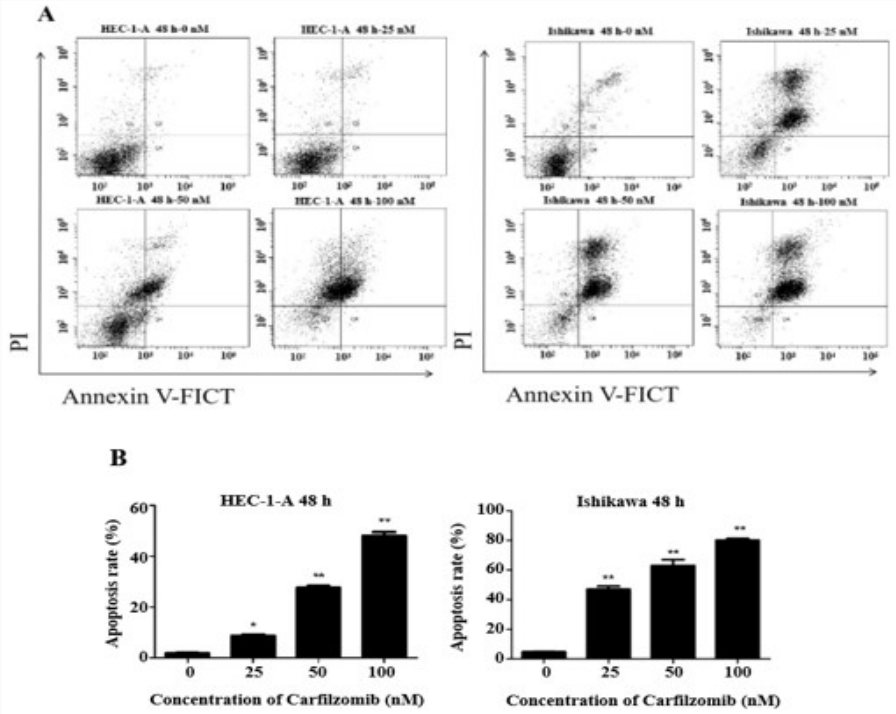

Figure 2: Effect of Carfilzomib on HEC-1-A and Ishikawa cell apoptosis after 48 h. (A) After being exposed to Carfilzomib for 48 h, Annexin V/PI staining was performed, and HEC-1-A and Ishikawa cells apoptosis was detected by flow cytometry. (B) Statistical chart of apoptosis rate of HEC-1-A and Ishikawa cells relative to the control group; *p<0.05, **p<0.01.

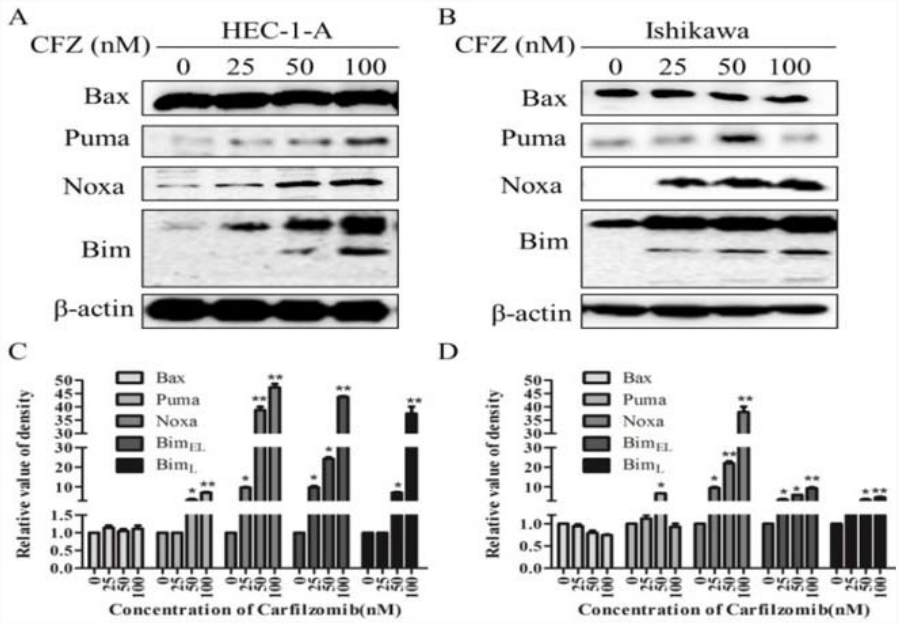

Figure 3: Effect of Carfilzomib on Bcl-2 apoptosis-promoting protein family in HEC-1-A and Ishikawa cells After 24 h of Carfilzomib treatment, the level of pro-apoptotic proteins in HEC-1-A (A) and Ishikawa (B) cells changed. (C, D) Relative quantitative diagram of protein in diagrams A (n=3) and B (n=3). Data are relative to the control group; *p<0.05, **p<0.01.

GRP78 dissociates from ER transmembrane proteins, leading to the polymerization of transmembrane proteins. Because *GRP78* is the main protective protein rapidly produced during ER stress, it is also recognized as a biomarker of ER stress. *CHOP* is a protein widely distributed in mammalian cells. It has an N-terminal activation domain and a C-terminal domain [13,14]. Normally, *CHOP* is rarely expressed and distributed in the cytoplasm. In the case of ER stress, *CHOP* is gradually upregulated and transferred to the nucleus. *CHOP* is an important intermediate signal molecule that plays a role in transcriptional regulation by forming heterodimers and combining with target genes. It is also one of the pathways involved in ER stress-induced apoptosis.

In this paper, based on previous studies on Carfilzomib, several tumor cell lines were treated with Carfilzomib drugs as in previous

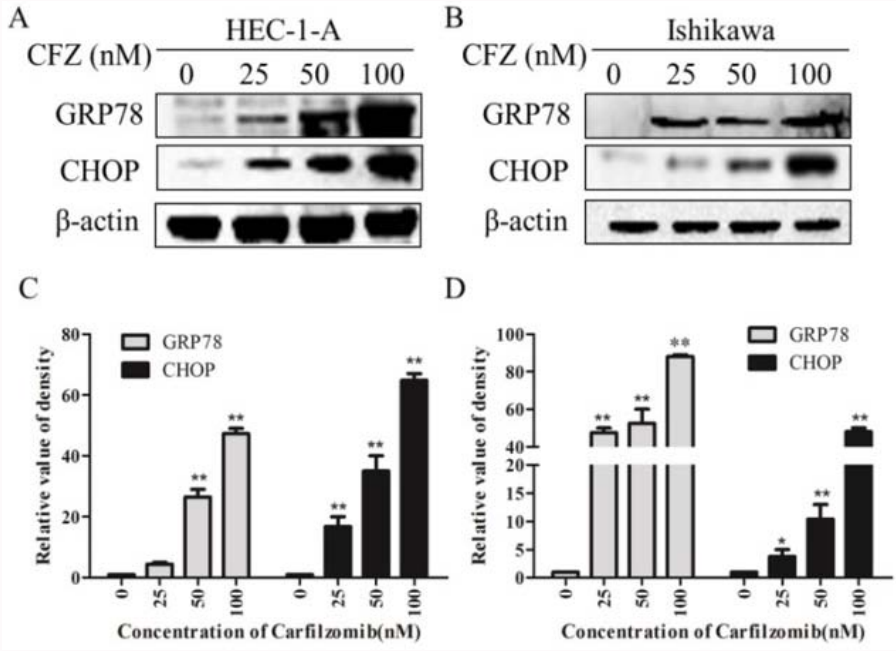

Figure 4: Effect of Carfilzomib on ER stress markers *GRP78* and *CHOP* in HEC-1-A. (A) and Ishikawa (B) cells. (C) Relative quantitative diagram of protein in diagram A (n=3) and (D) diagram B (n=3). Data are relative compared with the control group; *p<0.05, **p<0.01.

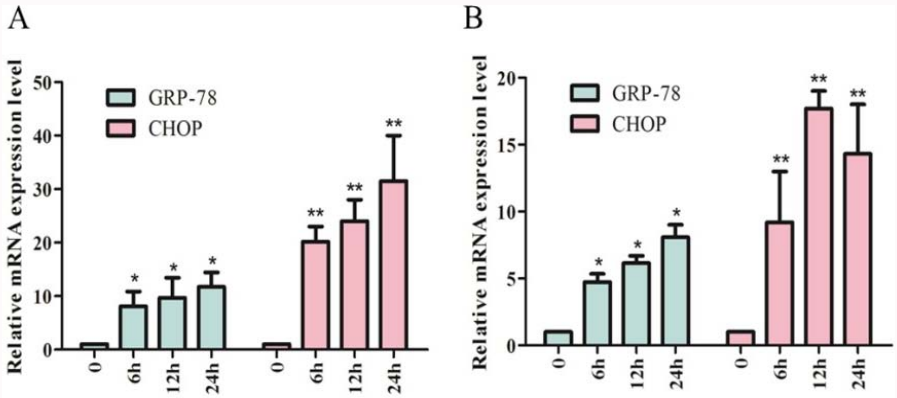

Figure 5: Effect of Carfilzomib on mRNA level of *GRP78* and *CHOP* in HEC-1-A and Ishikawa cell lines *GRP78* and *CHOP* mRNA expression changes in HEC-1-A (A) and Ishikawa (B) cells at different times after administration. Data are compared with the control group; * p<0.05, **p<0.01.

studies, and the results revealed that the growth inhibition rate of Carfilzomib on HEC-1-A and Ishikawa endometrial cancer cells was the highest by MTT screening. Therefore, two endometrial cancer cell lines were used for further experiments. Quantitative PCR and Western blot were used to detect the effect of Carfilzomib on *GRP78* and *CHOP* in the two endometrial cancer cell lines. Carfilzomib significantly increased the mRNA and protein expression of *GRP78* and *CHOP* genes.

The continuous effect of Carfilzomib causes severe ER stress in cells, which may also trigger cells to initiate endogenous mitochondrial pathway-mediated apoptosis. This study found that Puma, Noxa, and Bim, BH3-only proteins known as apoptosis-promoting factors, are triggered by ER stress and mediate apoptosis.

The experimental study on the expression changes of various signal factors in the ER stress signal pathway is required in our further study. And the molecular mechanism of how ER stress system starts the endogenous mitochondrial pathway to induce apoptosis, specifically, the change in PERK/eIF2 α and ATF6, as well as IRE1 α/*XBP1* path was will be our further research topics.

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