Original Article
Carfilzomib inhibits cell cycle progression at G2/M phase and induces apoptosis in breast cancer cells

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Abstract: Background: Carfilzomib is a second-generation proteasome inhibitor. Lately, it is approved for clinical treatment of multiple myeloma. However, its anti-tumor activity and mechanism against solid tumors has not been elucidated. The study is aimed to investigate the function and the mechanism of Carfilzomib against breast cancer cells. Methods: Breast cancer cell line ZR-75-30 was treated by different concentrations of Carfilzomib. MTT assay was used to examine cell viability. Colony formation assay was performed to detect cell proliferation. Flow cytometry was used to analyze cell cycle and apoptosis, and Hoechst 33258 staining was additionally used to observe cell apoptosis. Western blot was used to detect cell cycle- and apoptosis-associated protein expression level. Results: Carfilzomib impaired viability and colony formation ability of ZR-75-30 breast cancer cell line in a dose-dependent manner. Carfilzomib treatment suppressed protein expression of G2/M checkpoint-specific cyclin-dependent kinase CDK1 ($P<0.05$) and CDK2 ($P>0.05$). The pro-apoptotic protein Bax was increased, while the anti-apoptotic proteins Bcl-2 and Mcl-1 were decreased in Carfilzomib-treated cells in a dose-dependent manner. Conclusion: Carfilzomib could inhibit breast cancer cells proliferation and induce cell apoptosis, indicating its potential use for clinical management of breast cancer.

Keywords: Carfilzomib, breast cancer, cell proliferation, cell cycle, apoptosis

Introduction

Breast cancer is the most common malignancy and the first leading cause of cancer-related deaths among females worldwide [1]. Breast cancer mainly contains ductal carcinomas and lobular carcinomas. About 80% invasive carcinoma is the infiltrating ductal type [2]. The morbidity of breast cancer is increasing [3] from 22.9% of invasive cancers in women in 2008 to 25.2% of cancers diagnosed in women in 2012 [4]. Breast cancer is usually treated with surgery, chemotherapy or radiation therapy. Recent years, there are more therapeutics used to manage the disease, including hormone blocking therapy and monoclonal antibodies, which are usually depend on the receptor status on breast cancer cell surface and in their cytoplasm and nucleus. There are three important receptors: estrogen receptor (ER), progesterone receptor (PR) and HER2, which divide breast cancer into distinct molecular sub-types:

ER positive luminal cancers with generally better prognosis, more aggressive HER2 positive cancers, and most aggressive triple-negative or basal-like cancers negative in the three receptors [5]. These molecular types are correlated with response to therapy and prognosis [6]. Lately, new therapies are emerging to effectively prolong the survival time and improve prognosis of patients.

Cancer cells, which are often genetically instable and rapidly proliferative, tend to be more dependent on the proteasome than normal cells for the need of removing aberrant intracellular proteins [7]. Thus, inhibition of the 26S proteasome become an attractive approach for anti-cancer therapy [8]. Studies have found that proteasome inhibitors selectively target cancer cells and disturb numerous cellular activities leading to cell death or making them more sensitive to chemotherapeutic agents in breast cancer [9, 10], prostate cancer [11], pancreatic

cancer [12], ovarian cancer [13] and hepatocellular carcinoma [14]. Carfilzomib is a second-generation proteasome inhibitor, approved by FDA in 2012 to be used as a single agent to treat multiple myeloma (MM) patients who had received at least two prior therapies. Carfilzomib can irreversibly inhibit the chymotrypsin-like active sites of the 20S proteasome with a minimal amount of peripheral neuropathy than its predecessor [15]. Accumulating evidence have indicated that Carfilzomib can be a potential option for the treatment of solid tumors.

In eukaryotes, the proteasome is involved in degradation of most unneeded or damaged intracellular proteins by proteolysis. It is present in mammals as the form of a cytosolic 26S proteasome, which consist of a 20S core particle and two 19S regulatory cap subunits [16]. The proteasome is usually tagged with ubiquitin, which is essentially involved in many cellular processes such as cell cycle and apoptosis [17].

Cell cycle progression is a series of ordered events of cyclin-dependent kinases (CDK) activated by sequenced cyclins. When a CDK-cyclin complex has finished its function, they are proteolyzed by proteasome to insure the directional progression of cell cycle. In particular, exit from mitosis requires the proteasome-dependent dissociation of the cyclin B/CDK1 complex in G2/M phase [18]. The proteasome also plays an important role in the apoptotic process [19]. Occurrence of apoptosis mainly depends on the proteolytic caspase cascade. Although studies have found that inhibition of proteasome promotes apoptosis in most cell types [20, 21], researchers consider the involvement of the proteasome in apoptosis is mainly through disrupting the regulated degradation of pro-growth proteins, such as NF-kB [22] and STAT1 [23].

Until now, the potential therapeutic effects of Carfilzomib on breast cancer remain unknown. In this study, we explored the cytotoxic activity and the mechanism of Carfilzomib on breast cancer cell line ZR-75-30, and found that Carfilzomib could induce cell cycle arrest and apoptosis. These results suggested that Carfilzomib might be used as a candidate therapeutics for breast cancer.

Materials and methods

Drugs and antibodies

Carfilzomib was purchased from LC Laboratories (Woburn, MA, USA). Carfilzomib was reconstituted in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mmol/L and stored at -20°C. The stock was diluted in medium just before used and made the concentration of DMSO lower than 0.1%. The primary antibodies were listed as blow: mouse anti-GAPDH, rabbit anti-Bax, rabbit anti-Bcl-2, rabbit anti-Mcl-1, rabbit anti-CDC2, rabbit anti-CCNB1, rabbit anti-CDK2 (all from Proteintech Group, Inc., Wuhan, China). The secondary antibodies were HRP-conjugated goat anti-rabbit or mouse antibodies from Protech (Proteintech Group, Inc., Wuhan, China).

Cell culture and treatment

Breast cancer cell lines MCF-7 and ZR-75-30 were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM (Sigma-Aldrich; Schnelldorf, Germany) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; Logan, UT, USA), 100 U/mL of penicillin, and 50 µg/mL of streptomycin at 37°C in 5% CO_2.

Cell viability

MTT assay was used to assess cell viability with monotetrazolium (Sigma-Aldrich; Schnelldorf, Germany) assays. Briefly, the cells were seeded into 96-well plates for 24 h, and treated with indicated concentration of Carfilzomib for another 24 h. 0.5 mg/ml MTT was added to each well. The cells were cultured at 37°C for 4 h, 150 µl DMSO was added, and the 490 nm wave-length absorption value was measured. All experiments were performed in triplicate and repeated three times.

Colony formation

Cells were trypsinized into a single cell suspension. A total of 400 cells were plated in each well of 12-well plates for 24 h, and kept for another 7 days in DMEM supplemented with 10% fetal bovine serum containing Carfilzomib to allow colony formation. The colonies were fixed in methyl alcohol and stained using 0.1%
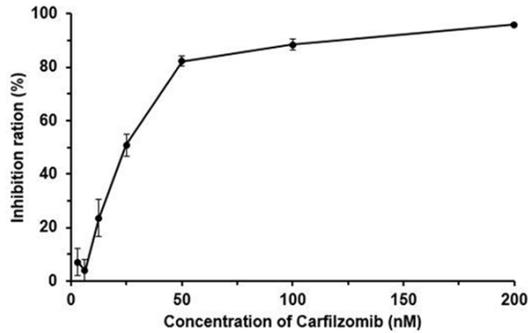
Figure 1. The inhibitory effect of Carfilzomib on viability of breast cancer cell lines. Cells were treated with indicated concentrations of Carfilzomib for 24 h. Carfilzomib impaired cell viability in a dose-dependent manner. And the inhibition on ZR-75-30 was more potent than MCF-7 cells. All experiments were performed in triplicate and every independent experiment was performed 3 times. Data were presented as mean ± SE.

crystal violet. Cell clones over 50 cells were counted using a grid. Three independent experiments were performed.

Cell cycle analysis

Cells were collected, washed twice with PBS, and fixed with ice-cold 70% ethanol for at least 1 h. The fixed cells were washed and stained with propidium iodide. After incubation for 30 min at 37°C, samples were analyzed by a flow cytometry (BD Biosciences; San Jose, CA, USA). Cell cycle analysis of DNA histograms was performed with CellQuest software.

Apoptosis assay

Apoptosis was first monitored by measuring nuclear condenses by Hoechst 33258 (Sigma-Aldrich; Schnelldorf, Germany) staining. Cells grown on glass cover slips were fixed with methyl alcohol for 20 min at room temperature, and then washed and stained with 167 mM Hoechst 33258 at 37°C for 30 min. Slides were observed using a fluorescence microscope.

Apoptosis was then quantified with Annexin V-FITC/PI double staining kit (Beyotime Institute of Biotechnology; Haimen, China). After 24 hours treatment with Carfilzomib, 1×10^6 cells were harvested and washed in PBS, resuspended in 500 µl of Annexin V binding buffer. Annexin V-FITC and propidium iodide (PI) were then added to the cells. The cell mixture was incubated in the dark at room temperature for

15 min according to the manufacturer's instructions. Apoptosis was measured using a flow cytometer (BD Biosciences; San Jose, CA, USA). Annexin V single positive cells were identified as early apoptotic cells. PI single positive cells were identified as necrotic cells. Annexin V/PI double positive cells were identified as late apoptotic cells. Furthermore, expression of pro-apoptotic Bax, and anti-apoptotic Bcl-2 and Mcl-1 proteins was detected with western blot.

Western blot

Cells were lysed with RIPA buffer containing protease inhibitor cocktail (Roche Applied Science; Mannheim, Germany) and PMSF. Protein concentration was determined by BCA protein assay reagent kit (Pierce; Rockford, IL, USA). Equal amounts of cell lysates were separated by 12% SDS-PAGE, electrophoretically transferred to PVDF membrane (Millipore Corporation; Bedford, MA, USA), immunoblotted with primary antibodies against Bax, Bcl-2, Mcl-1, CDC2, CCNB1, CDK2 or GAPDH. Blots were visualized with anti-rabbit or anti-mouse IgG conjugated with peroxidase (HRP) and ECL reagents (Millipore Corporation; Bedford, MA, USA).

Statistical analysis

Data were presented as the means ± SE and were analyzed using SPSS 22.0 software (Chicago, IL, USA). Statistical differences were tested by two-tailed t-test. Differences were considered significant at P<0.05 (*) or highly significant at P<0.001 (**).

Results

Carfilzomib impaired cell viability

MTT assay showed that 24 h-treatment of Carfilzomib reduced cell viability in a concentration-dependent manner. 3.125 nM and 6.250 nM of Carfilzomib did not cause obvious cell death. The suppressive effect of Carfilzomib on cell viability became significant at concentrations higher than 12.500 nM, which reached a plateau at concentrations higher than 100.0 nM (**Figure 1**).

Carfilzomib reduced colony formation ability

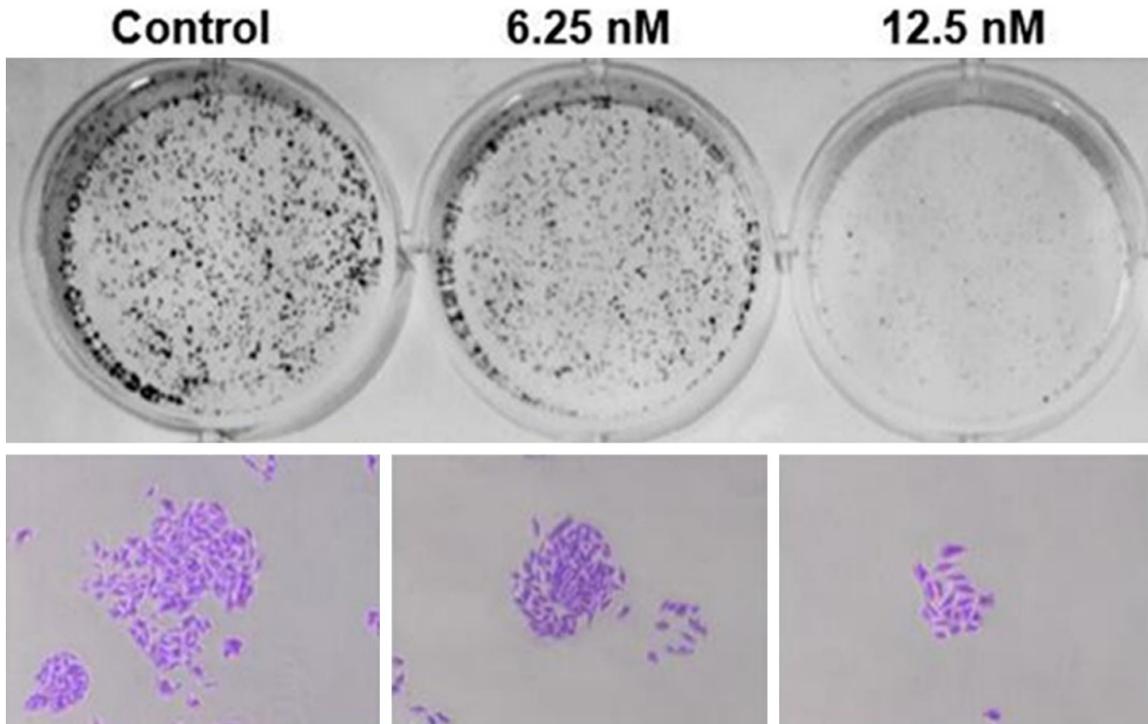In addition to reduce cell proliferation, Carfilzomib also decreased colony formation ability

Figure 2. The suppression of Carfilzomib on proliferation of breast cancer cells. ZR-75-30 cells were treated with indicated concentrations of Carfilzomib for 7 days. Carfilzomib repressed cell colony formation ability in a dose-dependent manner. No visible colonies were observed for cells treated by 25 nM Carfilzomib (100×). All experiments were performed in triplicate and every independent experiment was performed 3 times. Data were presented as mean ± SE.

of ZR-75-30 cells in a concentration-dependent manner. After 7-day culture, not only the number of colony but also the cell numbers in each colony were significantly lessened in all Carfilzomib-treated groups relative to non-treated control group (**Figure 2**). And no colony was formed in 25 nM Carfilzomib treated group (data not shown).

Carfilzomib arrested cell cycle progression at the G2/M phase

We subsequently assessed cell cycle progression by flow cytometry. Compared to the non-treated control cells, 25 nM of Carfilzomib induced a significant increase in the relative number of cells in G2/M-phase (73.10 ± 1.273% vs 10.90 ± 3.811%), and a significant decrease in G0/G1-phase cells (20.38 ± 4.900% vs 68.37 ± 2.249%). Decrease of cells number in S-phase were observed in 25 nM Carfilzomib-treated group, but the differences were not significant (**Figure 3A** and **3B**).

We then examined the expression of molecules essential for G2/M phase transition of cell cycle. As **Figure 3C** showed, 25 nM Carfilzomib treatment suppressed protein expression of G2/M checkpoint-specific cyclin-dependent kinase CDC2 ($P < 0.05$) and CDK2 ($P > 0.05$), while no reduction of cyclin CCNB1 was observed (**Figure 3C** and **3D**).

Carfilzomib induced cell apoptosis

Hoechst 33258 staining was used to detect cell apoptosis. Most of nuclei in non-treated cells displayed dispersal uniform fluorescence which indicated normal nuclei, suggesting few apoptosis occurred in negative control cells. Contrarily, dense staining of Hoechst 33258, indicating chromatin condensation in the nuclei of apoptotic cells, was observed in Carfilzomib-treated cells, especially in cells treated by 25 nM of Carfilzomib (**Figure 4A** and **4B**).

To further evaluate the effects of Carfilzomib on ZR-75-30 cells apoptosis, we performed Annexin V-FITC and PI fluorescence staining. Annexin V-FITC and PI signals could barely be detected in blank control cells, while a significant increase in fluorescence densities were visible
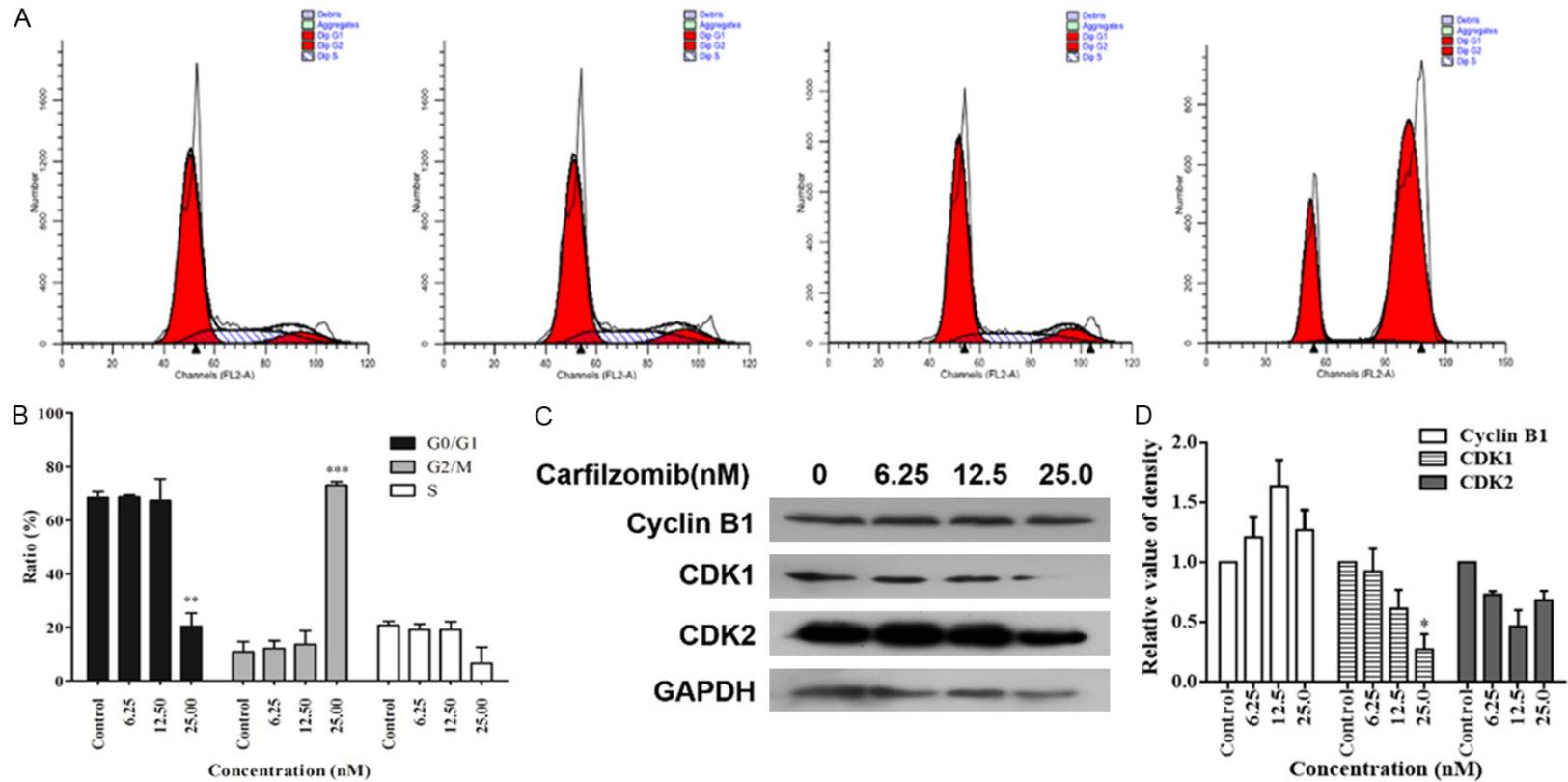
Figure 3. The effect of Carfilzomib on cell cycle profile of ZR-75-30 cells. A and B: ZR-75-30 cells were incubated with indicated doses of Carfilzomib for 24 h, and significant G2/M arrest was observed by flow cytometry in cell treated by 25 nM of Carfilzomib. Every independent experiment was performed 3 times. C and D: The expression of cell cycle regulatory proteins in Carfilzomib-treated ZR-75-30 cells. Western blot results showed that CDK 1 and 2 were obviously decreased in 25 nM Carfilzomib-treated ZR-75-30 cells. But no obvious change was found in Cyclin B1.
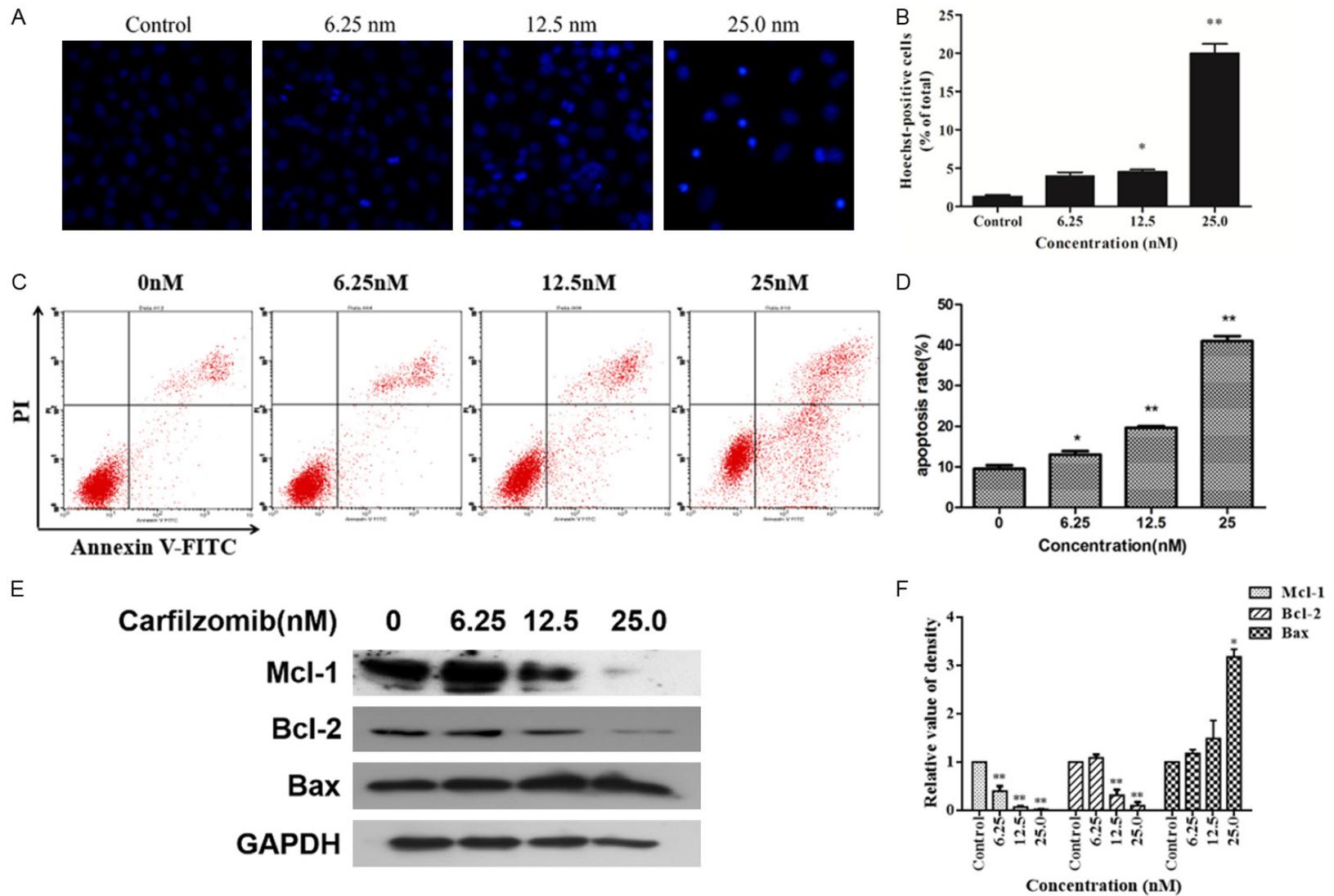
Figure 4. Carfilzomib induced cell apoptosis. A and B: Annexin V-FITC and PI fluorescence staining combined with flow cytometry showed that the percentages of apoptotic cells were increased from 9.5% in non-treated negative control cells, 13.0% in 6.25 nM Carfilzomib-treatedZR-75-30 cells, 19.6% 12.5 nM Carfilzomib-treated cells, to 41.0% in 25 nM Carfilzomib-treated cells. C and D: Dense staining of Hoechst 33258 was observed in nuclei of Carfilzomib- treated cells, which was increased along with the addition of Carfilzomib concentration. E and F: The expression of Bax, Bcl-2 and Mcl-1 in Carfilzomib-treated ZR-75-30 cells. Western blot results showed that Bax increased in Carfilzomib-treated ZR-75-30 cells in a dose-dependent manner, while Bcl-2 and Mcl-1 decreased in 12.5 and 25 nM Carfilzomib-treated ZR-75-30 cells.

in response to 6.25 nM (P<0.05), 12.5 nM (P<0.01) and 25 nM (P<0.01) of Carfilzomib treatment for 24 h. These results suggested that Carfilzomib was capable of inducing early apoptosis and late apoptosis in breast cancer cells in a dose-dependent manner (**Figure 4C** and **4D**).

We further examined the expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 and Mcl-1. Bax was increased while Bcl-2 and Mcl-1 were decreased in a Carfilzomib concentration dependent manner (**Figure 4E** and **4F**). These data therefore demonstrated that the Carfilzomib induced apoptosis in breast cancer cells.

Discussion

Carfilzomib, a second-generation proteasome inhibitor, is a cell-permeable tetrapeptide epoxyketone analog of epoxomicin. Although Carfilzomib is highly effective against multiple myeloma, its anti-tumor activity and mechanism largely remain unclear in breast cancer. In the present study, Carfilzomib was found to exert a cytotoxicity effect on breast cancer cell ZR-75-30 through arresting cell cycle at G2/M phase and inducing apoptosis.

Targeting the proteasome represents a novel treatment emerging recent years. Since the first proteasome inhibitor Bortezomib presents remarkable clinical success in the treatment of multiple myeloma, the proteasome inhibitor has been widely investigated in solid tumor therapy. Aras B et al found that Bortezomib combined with Etoposide exert synergistic effects on the human prostate cancer cell line PC-3. But Bortezomib has its inherent defect that it induces severe and dose-limiting peripheral neuropathy which is most likely due to cross-inhibition with HtrA2/Omi, a neuronal serine protease implicated in neuron survival [24]. More efforts are made to produce new forms of proteasome inhibitor to enhance the efficacy and reduce the side effect. Carfilzomib is the next-generation proteasome inhibitor with higher specificities and reduced toxicities. It can irreversibly inhibit the chymotrypsin-like active sites of the 20S proteasome with a minimal amount of peripheral neuropathy than its predecessor. The Food and Drug Administration (FDA) has approved Carfilzomib for the treatment of multiple myeloma in 2012 [25]. Accumulating evidences indicate that Carfilzomib could be a possible therapy for the treatment of solid tumors including breast cancer, but the mechanisms remain unknown. Hao Wang et al have found that the proteasome inhibitor MLN9708 sensitizes breast cancer cells to doxorubicin-induced apoptosis [10]. Y GU and Bouwman et al have found that Bortezomib inhibits cancer cells growth while suppression of BRCA1 sensitizes cells to proteasome inhibitors via deregulating cell cycle checkpoints mediated by RB1-E2F [26]. We hypothesize that the new generation proteasome inhibitor Carfilzomib may play the similar role in breast cancer via impacting cell cycle and apoptosis.

It is found that Carfilzomib reduces cell viability and proliferation in a dose-dependent manner via downregulating G2/M checkpoint-specific cyclin-dependent kinase CDK1 and CDK2 to arrest cell cycle at G2/M-phase, and induces cell apoptosis via increasing pro-apoptotic protein Bax while decreasing the anti-apoptotic proteins Bcl-2 and Mcl-1. Our data lay the foundation for necessary additional studies of proteasome inhibitors to warrant its clinical use as a new anti-cancer drug for the treatment of various solid tumors including breast cancer.

Disclosure of conflict of interest

None.

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