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Original Article

Carfilzomib induces G2/M cell cycle arrest in human endometrial cancer cells via upregulation of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$

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ABSTRACT

Objective: Carfilzomib is a second-generation tetrapeptide epoxyketone proteasome inhibitor used in current clinical therapy of hematologic malignancies. The mechanism of proteasome inhibition in endometrial cancer is not very clear. Carfilzomib inhibition of type I endometrial carcinoma cell proliferation by inducing cell cycle arrest at the G2/M phase was investigated in our study.

Materials and methods: HEC-1-A and Ishikawa endometrial carcinoma cell lines and three tumor cell lines were treated by different concentrations of carfilzomib. Methyl thiazolyl tetrazolium (MTT) assay was used to detect cell viability. Flow cytometry was used to analyze the cell cycle. Western blot was used to detect proteins involved in cell cycle progression.

Results: Carfilzomib impaired viability of myelogenous leukemia cell line K562, cervical cancer cell line HeLa, hepatocellular carcinoma cell line SMCC-7721, and endometrial carcinoma cell lines HEC-1-A and Ishikawa. The cell cycle was arrested at the G2/M phase in carfilzomib-treated HEC-1-A endometrial carcinoma cells, while it was arrested at both S and G2/M phases in carfilzomib-treated Ishikawa cells. Carfilzomib treatment significantly induced $p21^{Waf1/Cip1}$ and p27, while substantially reduced cyclin D3 and cyclin-dependent kinase 1.

Conclusion: This study showed that carfilzomib inhibited endometrial cancer proliferation by upregulating cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, and reducing cyclin-dependent kinase 1 to arrest the cell cycle at the G2/M phase.

Introduction

Endometrial carcinoma (EC) is the most common gynecological cancer in developed countries, and its prevalence is increasing [1]. Generally, endometrial cancers are classified into two types. Type I ECs account for ~80% of ECs, comprising low-grade endometrial cancers and are often associated with estrogen. The main risk factor of type I ECs is exposure to endogenous and exogenous estrogens associated with obesity, diabetes, early age at menarche, nulliparity, late-onset menopause, older age, and use of tamoxifen [2]. Type II ECs comprise a group of high-risk tumors of serous, clear cell, or poorly differentiated endometrioid-type ECs that are not estrogen driven. Despite its high incidence, the prognosis of type I ECs is better than that of ovarian and cervical cancers. Presumably because the disease is frequently symptomatic at an early stage, most patients with a symptom of irregular vaginal bleeding are diagnosed when the lesions are confined in uterus, so that the 5-year survival rate is up to 90% after surgery [3]. However, there are still 15–25% patients with advanced stage or recurrence that have no obvious improvement because of lack of new therapeutic methods besides surgery, radiotherapy, and traditional chemotherapy. Therefore, exploring new antitumor therapeutics of high sensitivity still has important clinical significance.

Carfilzomib, a second-in-class proteasome inhibitor, is approved by the Food and Drug Administration for the treatment of multiple myeloma in 2012 [4]. It is a synthetic tetrapeptide epoxyketone proteasome inhibitor that irreversibly binds to the amino terminal catalytic Thr-1 of 20S proteasome causing sustained proteasome

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inhibition [5]. The proteasome is a large multisubunit protease complex, which is an important component of the ubiquitin–proteasome system to mediate protein degradation in cells of species ranging from archaebacteria and yeast to humans. Since the proteasome plays a fundamental role in protein homeostasis and regulates many cell survival events including proliferation, apoptosis, and carcinogenesis, it has emerged as a promising cancer therapeutic target. In recent years, the antitumor activity of proteasome inhibitor has been a hot topic in cancer research. It is reported that rapidly proliferating cancer cells are more sensitive to proteasome inhibitors than normal cells [6]. Researchers have developed a variety of proteasome inhibitors. In contrast to the first-generation proteasome inhibitor bortezomib, carfilzomib can irreversibly bind and inhibit the chymotrypsin-like site of the proteasome to kill the cancer cells more efficiently with fewer adverse effects, especially the neurotoxicity associated with bortezomib. Currently, carfilzomib was found to have antitumor activity in hematological cancer [7,8] and many other solid cancers, including head and neck squamous cell carcinoma [9], glioblastoma [10], colorectal cancer [11], and lung cancer [12]. However, its role in EC has not yet been discussed.

The cell cycle is a physiological process that refers to the series of events that take place in a cell division. This process is under precise control to regulate cell proliferation, apoptosis, and other events crucial for cell survival [13]. The molecular events that control the cell cycle are ordered and directional. It is well known that the cell cycle process is controlled by a series of cyclin-dependent kinases (CDKs), which is activated by cyclins. Cyclin D is the first cyclin produced in the cell cycle; it binds to CDK4 to form an active cyclin D/CDK4 complex that phosphorylates the retinoblastoma susceptibility protein (Rb), then activates E2F, and finally results in transcription of various genes to prepare for G1/S phase transformation, including cyclin E [14]. Cyclin E thus binds to CDK2 and promotes cell cycle progression from the G1 to the S phase [15]. Cyclin B activates CDK1 (also known as CDC2) to facilitate the completion of mitosis [16]. In addition, inhibitors of CDKs, the cip/kip family and the INK4a/ARF family, prevent the progression of the cell cycle. A member of the cip/kip family p27 halts the cell cycle by binding to CDKs. Another cip/kip family member p21, activated by p53, inactivates CDKs to arrest the cell cycle. The INK4a/ARF family, including $p16^{INK4a}$ and $p14^{INK4a}$, competitively binds to CDK4/6 to prevent its combination with cyclin D [17]. It is reported that almost all cancers globally present the deficiency of cell cycle regulation. Dysregulation of the cell cycle may induce tumor formation from promoting cell malignant proliferation and failing to respond DNA damage. Recently, many studies have demonstrated that cell cycle checkpoint regulation plays an important role in tumor progression, and that many drugs exert their antitumor effect through regulating the cell cycle, such as paclitaxel, which is a first-line chemotherapy drug for breast cancer and ovarian cancer [18]. Thus, exploring the mechanism of cell cycle dysregulation and the potential therapeutic targets are of great significance for the treatment of cancer.

In this study, we examined the effects of carfilzomib on the inhibition of proliferation of EC cell lines, and investigated the mechanism of carfilzomib-induced cell cycle arrest, particularly in EC cells. It suggested that carfilzomib may provide a possible drug therapeutic approach for EC.

Materials and methods

Drugs and antibodies

Carfilzomib was acquired from LC Laboratories (Woburn, MA, USA). Carfilzomib was reconstituted in dimethyl sulfoxide at a stock concentration of 10 mmol/L and stored at −20°C until use. The stock was diluted in a medium to the indicated concentrations just before use, and the concentration of dimethyl sulfoxide was made lower than 0.1%. The primary antibodies were as follows: rabbit anti-cyclin B1 and p27Kip1 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-cyclin D3, cyclin E1, CDK1, CDK2, CDK4, $p21^{Waf1/Cip1}$, and mouse anti-β-actin (Proteintech Group, Inc., Wuhan, China). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse antibodies from Proteintech Group, Inc.

Cell culture

Myelogenous leukemia cell line K562, and EC cell lines HEC-1-A and Ishikawa were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, Schnelldorf, Germany) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, and 50 g/mL streptomycin at 37°C in 5% CO_2.

Cell viability assay

Exponentially growing cells were seeded in 96-well plates for 24 hours after seeding, cells were incubated in the absence or presence of carfilzomib for 24 hours. The cell viability was evaluated using MTT assay. Cell numbers were analyzed for the dynamic proliferation rate of cells. The experiment was repeated three times independently.

Cell cycle analysis

HEC-1-A and Ishikawa cells were cultured in six-well culture plates for 24 hours, and then the medium was changed to a serum-free medium. Cells were incubated with carfilzomib for another 24 hours. The cells were trypsinized, collected, and washed in phosphate-buffered saline; the cells were subsequently fixed in 70% ethanol, incubated with RNase (50 μg/mL) and propidium iodide (PI) (60 μg/mL), and protected from light at room temperature for 30 minutes. The cell cycle was assessed according to the percentage of cells with DNA using the PI staining technique as described previously.

Western blot

The proteins of HEC-1-A and Ishikawa cells treated with or without carfilzomib for 24 hours were extracted with radio immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail on ice. Lysates were resolved on 10% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a poly vinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 hour at room temperature with 5% nonfat milk, and then incubated with primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 hour. The protein band was detected using the electro-chemi-luminescence (ECL) reagents (Millipore Corporation; Bedford, MA, USA).

Statistical analysis

Data were analyzed using SPSS 22.0 software (SPSS Inc., 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 inhibits cell viability of tumor cell lines

To evaluate the antitumor activity of carfilzomib, we first tested the efficacy of carfilzomib on cell viability of tumor cells *in vitro*. As shown in Figure 1, inhibitory activity of carfilzomib against cell viability in a dose-dependent manner was observed in three tumor cell lines, including K562, and EC cells HEC-1-A and Ishikawa. HEC-1-A cells were more sensitive to carfilzomib than Ishikawa cells. The inhibitory effect on HEC-1-A cell viability was comparable with K562 myelogenous leukemia cell viability. The results indicated that carfilzomib could impair viability of both blood tumor cells and solid tumor cells, and it could be used to suppress EC proliferation.

Effect of carfilzomib on EC cell cycle profile

As carfilzomib inhibited EC cell viability, we next investigated its effect on EC cell cycle progression. Significant G2/M phase arrest was induced in HEC-1-A cells treated by 50nM and 100nM of carfilzomib (Figure 2A), while 25nM of carfilzomib easily caused significant G2/M phase arrest in Ishikawa cells. In addition, substantial S phase arrest was observed in Ishikawa cells treated by 50nM and 100nM of carfilzomib (Figure 2B). The results indicated that carfilzomib suppressed EC proliferation by inhibiting cell cycle progression.

Effect of carfilzomib on the expression of cell cycle regulatory proteins

To further investigate the role of carfilzomib in the regulation of cell cycle progression, expression of cell cycle regulatory proteins was examined with Western blot. In both EC cell lines, 50nM and 100nM of carfilzomib obviously decreased protein level of cyclin D3 (Figure 3). However, no obvious change of cyclin B1, cyclin E1, CDK1, CDK2, and CDK4 was found in carfilzomib-treated cells (Figure 3).

To understand the mechanism of G2/M phase arrest induced by carfilzomib, changes in the expression of CDK inhibitors $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ in carfilzomib-treated cells were then detected. Western blot results showed that 50nM and 100nM of carfilzomib substantially increased the expression of $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ in both EC cell lines (Figure 4). In Ishikawa cells, upregulation of $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ was also observed in cells treated by 25nM of carfilzomib. Since $p21^{Waf1/Cip1}$ was activated by p53, we compared p53 protein level in cells treated and not treated by carfilzomib. However, no obvious change of p53 was found in carfilzomib-treated cells relative to nontreated controls. In all, carfilzomib could retard EC cell growth via upregulation of $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ independent of p53 to arrest cell cycle progression mainly at the G2/M phase.

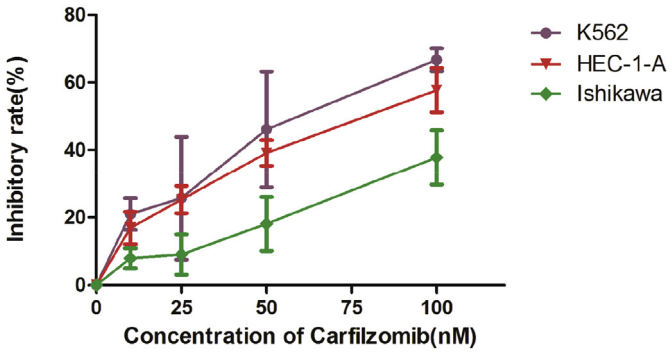

Figure 1. The inhibitory effect of carfilzomib on the viability of five tumor cell lines. Cells were treated with indicated concentrations of carfilzomib for 24 hours. Carfilzomib impaired cell viability in a dose-dependent manner. The inhibition on solid tumor cells, especially HEC-1-A endometrial carcinoma cells, was comparable with K562 myelogenous leukemia cells. All experiments were performed in triplicate and every independent experiment was performed three times. Data were presented as mean ± SE. SE = standard error.

Discussion

The second-generation proteasome inhibitor carfilzomib is a cell-permeable tetrapeptide epoxyketone analog of epoxomicin, which is highly effective in multiple myeloma patients. It is still unknown whether it is effective in solid tumor patients. The present study is focused on the inhibitory effect of carfilzomib on type I ECs.

HEC-1-A and Ishikawa cell lines were used to study the mechanisms of the effect of carfilzomib on type I ECs. It was found that carfilzomib inhibited the viability of EC cells in a dose-dependent manner. Cell cycle analysis showed that carfilzomib altered cell cycle distribution by decreasing the proportion of cells in the G1 phase and increasing the proportion in the S and G2/M phases. The cell cycle is regulated by specific cyclins and CDKs in an orderly manner. Cancer cells are characterized by uncontrolled proliferation due to dysregulation of the cell cycle. Different antitumor therapeutics are, therefore, designed to kill malignant cells by controlling the cell cycle. Here we found that carfilzomib exposure led to a significant decrease of cyclin D3 and increase of $p27^{Kip1}$ and $p21^{Waf1/Cip1}$. The cyclin kinase inhibitor $p21^{Waf1/Cip1}$ can promote cell cycle arrest in G1/S and G2/M transitions by inhibiting cyclin D/CDK4,6 and cyclin-E/CDK2, respectively, in response to a variety of stimuli; p53 is the main transcriptional regulator of $p21^{Waf1/Cip1}$, which contains two conserved p53-responsive elements in its promoter [19]. Various stresses including DNA damage and oxidative stress activate p53 and subsequently result in $p21^{Waf1/Cip1}$ expression [20]. However, recent evidence also indicates that the induction of $p21^{Waf1/Cip1}$ may be independent of the p53 pathway [21–23]. Our study showed that $p21^{Waf1/Cip1}$ was significantly induced in two EC cell lines and carfilzomib had no effect on the expression of p53 protein, suggesting that the $p21^{Waf1/Cip1}$ upregulation was independent of the p53 pathway (Figure 4). Similarly, it was found that PS-341-induced G2/M phase arrest was associated with the upregulation of $p21^{Waf1/Cip1}$ via a p53-independent pathway [24].

Currently, there are no reports on the mechanism of carfilzomib treatment of type I ECs. Our study shows that carfilzomib could suppress EC cell proliferation by upregulating CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, and downregulating cyclin D3 to block the cell cycle at the G2/M phase. As type I ECs are estrogen-induced cancers, Pavlides et al [25] have found that mitogenic 17-β-estradiol (E2) induces degradation of p27 by E3 ligase complex in primary endometrial epithelial cells and EC cell lines, suggesting that proteasome inhibitor carfilzomib prevents the degradation of p27 by E3 ligase complex and thus prevents estrogen-induced proliferation. However, more experiments should be conducted to prove the assumption.

Collectively, we found that carfilzomib could suppress EC cell proliferation via upregulation of CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ to arrest the cell cycle at the G2/M phase that was independent of p53. Although the mechanisms of carfilzomib-induced changes of cyclin D3, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ need further investigation, our results indicate that carfilzomib might be a potential agent against EC.

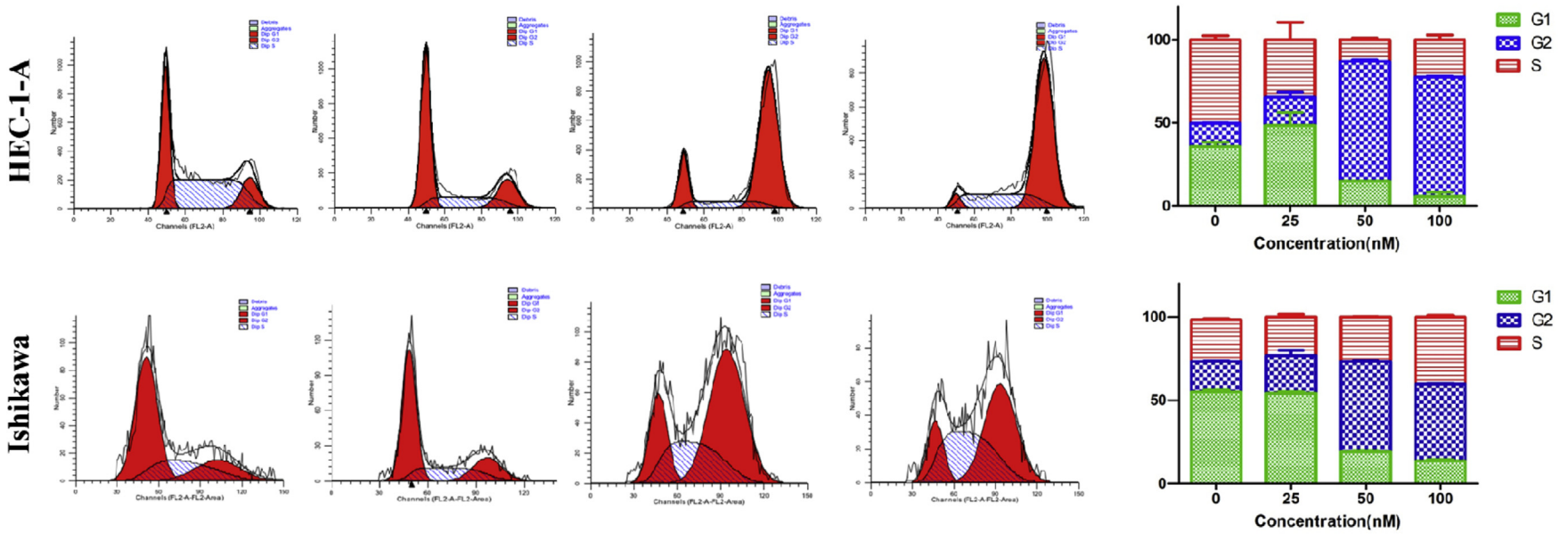

Figure 2. Effect of carfilzomib on EC cell cycle profile. (A) HEC-1-A cells were incubated with indicated doses of carfilzomib for 24 hours. Carfilzomib (50nM and 100nM) caused significant G2/M phase arrest. (B) Ishikawa cells were incubated with indicated doses of carfilzomib for 24 hours. Carfilzomib at 25nM induced G2/M arrest, while 50nM and 100nM carfilzomib caused significant G2/M and S phase arrest. Every independent experiment was performed three times. The unit nM indicates nmol/L. EC = Endometrial carcinoma.

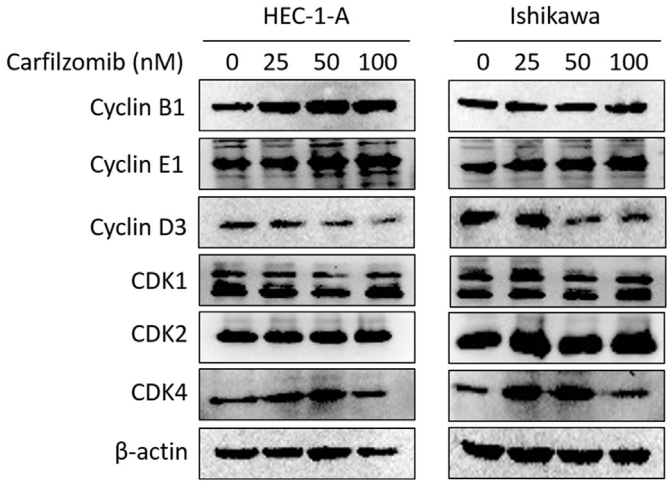

Figure 3. Expression of cell cycle regulatory proteins in carfilzomib-treated HEC-1-A and Ishikawa cells. Western blot results showed that cyclin D3 was decreased in 50nM and 100nM carfilzomib-treated HEC-1-A and Ishikawa cells. However, no obvious change was found in other cyclins and CDKs. CDK = cyclin-dependent kinase.

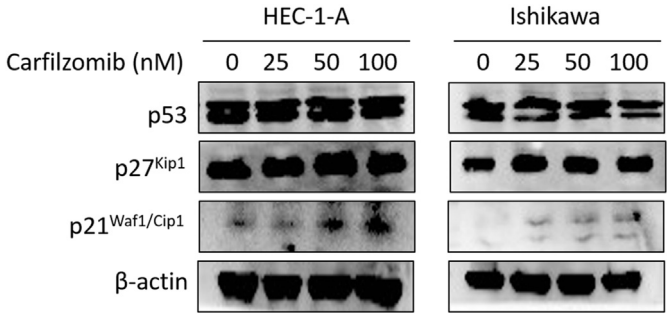

Figure 4. Expression of $p27^{Kip1}$, $p21^{Waf1/Cip1}$, and p53 in carfilzomib-treated HEC-1-A and Ishikawa cells. Western blot results showed that $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ were increased in 50nM and 100nM carfilzomib-treated HEC-1-A and Ishikawa cells. Increases of $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ were observed in 25nM carfilzomib-treated Ishikawa cells rather than in HEC-1-A cells. However, p53 expression level remained almost unchanged in carfilzomib-treated HEC-1-A and Ishikawa cells.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

None.

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