In the present study, we investigated the interactions between proteasome inhibitor carfilzomib (CFZ) and histone deacetylase inhibitor vorinostat in Jurkat T-leukemia cells. Coexposure of cells to minimally lethal concentrations of CFZ with very low concentration of vorinostat resulted in synergistic antiproliferative effects and enhanced apoptosis in Jurkat T-leukemia cells, accompanied with the sharply increased reactive oxygen species (ROS), the striking decrease in the mitochondrial membrane potential (MMP), the increased release of cytochrome c, the enhanced activation of caspase-9 and -3, and the cleavage of PARP. The combined treatment of Jurkat cells pre-treated with ROS scavengers N-acetylcysteine (NAC) significantly blocked the loss of mitochondrial membrane potential, suggesting that ROS generation was a former event of the loss of mitochondrial membrane potential. Furthermore, NAC also resulted in a marked reduction in apoptotic cells, indicating a critical role for increased ROS generation by combined treatment.

In addition, combined treatment arrested the cell cycle in G2-M phase. These results imply that CFZ interacted synergistically with vorinostat in Jurkat T-leukemia cells, which raised the possibility that the combination of carfilzomib with vorinostat may represent a novel strategy in treating T-cell Leukemia.

Keywords carfilzomib; vorinostat; reactive oxygen species; apoptosis; Jurkat

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignant tumor and has a poor prognosis. T-ALL accounts for 15% of newly diagnosed ALL cases in children and 25% in adults [1,2]. Conventional chemotherapy was unsatisfactory for T-All, with a minimal effect on survival or quality of life [3,4]. Therefore, new, effective, and well-tolerated therapy strategies are urgently needed. At present, these agents targeting cell signaling pathways regarding the pathopoiesis and development of T cells have been regarded as a promising treatment option for T-ALL [5].

Carfilzomib (CFZ) is a second-generation irreversible proteasome inhibitor which has preclinical activity against bortezomib-resistant cells [6] and it has been shown that single agent or combination with other agents is useful in the treatment of multiple myeloma [7] and some other cancers [8]. CFZ has been approved for the treatment of multiple myeloma [9].

Histone deacetylase inhibitors increase gene expression through induction of histone acetylation [10]. Histone deacetylase inhibitors alter the cell cycle distribution and induce cell death, apoptosis, and differentiation in malignant and transformed cells [11]. Histone deacetylase inhibitor vorinostat has been approved for the treatment of cutaneous T-cell lymphoma by the US Food and Drug Administration [12].

First-in-class proteasome inhibitor bortezomib and histone deacetylase inhibitors have been reported to have synergistic interactions in some solid tumors [13,14] and hematologic tumors [15,16]. CFZ is a second-generation irreversible proteasome inhibitor and has shown activity against bortezomib-resistant cells, indicating that their mechanisms of action are not completely identical [7]. Combined treatment with the second-generation proteasome inhibitor CFZ and histone deacetylase inhibitor vorinostat has currently been reported mainly in non-Hodgkin’s lymphoma [17,18]. The purpose of the present study was to investigate whether the combination of CFZ and vorinostat exerts synergistic activity in Jurkat T-leukemia cells. Herein, we reported a novel mechanism of action of the combination of proteasome inhibitor CFZ with
histone deacetylase inhibitor vorinostat, and suggested that this combined treatment can be used as a new strategy for T-ALL treatment.

**Materials and Methods**

**Reagents**

CFZ was purchased from Onyx Pharmaceuticals (South San Francisco, USA). Vorinostat was purchased from Merck & Co., Inc. (Rahway, USA). These agents were formulated in dimethyl sulfoxide. N-acetyl-l-cysteine (NAC) was purchased from Sigma-Aldrich (St Louis, USA) and dissolved in ddH2O. The Cell Counting kit-8 (CCK-8) was purchased from Dojindo (Mashikimachi, Japan). The Cell Apoptosis kit was purchased from BD Pharmingen (Franklin Lakes, USA). The Mitochondrial Membrane Potential Assay kit with JC-1 and Cell Mitochondria Isolation kit were purchased from Beyotime Institute of Biotechnology (Haimen, China).

**Cell culture**

Jurkat cells were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences (Shanghai, China). The cell line was maintained in RPMI-1640 medium (Invitrogen, Frederick, USA), supplemented with 10% fetal bovine serum, 1% penicillin (100 units/ml), and 1% streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO2 and 95% air.

**Assessment of cell death and apoptosis**

Cell proliferation was evaluated by Cell Counting kit-8. Cell apoptosis was measured by Annexin V-FITC and propidium iodide (PI) staining (BD Pharmingen). In our studies, apoptotic cells included early (Annexin V+/PI−) and late (Annexin V+/PI+) apoptosis. Results of CCK-8 and Annexin V-FITC and PI assays were concordant.

**Cytosolic fractions**

Cytosolic fractions were obtained using a Cell Mitochondria Isolation kit (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions and used for western blot analysis of cytochrome c.

**Western blot analysis**

The cells were lysed in lysis buffer (1.0% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM PMSF) on ice for 30 min, then the supernatant was separated and collected by centrifugation. Equal amounts of proteins (30 μg per lane) were separated on 10% or 15% SDS–PAGE, transferred to nitrocellulose membrane, blocked for 1 h with 5% skim milk, and probed with primary antibodies overnight at 4°C. Primary antibodies were as follows: anti-cytochrome c, anti-caspase-9, anti-caspase-3, anti-PARP, and anti-β-actin antibodies. All were from Cell Signaling Technology (Beverly, USA). Fluorescence-conjugated goat anti-mouse IgG was used as the secondary antibody. Fluorescence was measured by Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, USA). The signal of β-actin was used as an internal control.

**Detection of reactive oxygen species**

Cells were pretreated with or without NAC at 37°C for 20 min, and were incubated with various drugs for indicated time, then the cells were washed with phosphate buffered saline (PBS) and were incubated with 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 20 min and the fluorescence intensity was assessed using a flow cytometer (BD, San Diego, USA).

**Analysis of mitochondrial membrane potential**

The change in mitochondrial membrane potential was measured by flow cytometry using JC-1 dye (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions.

**Cell cycle analysis**

Cells were collected and washed with ice-cold PBS, fixed in 70% ethanol at −20°C for 16 h, and stained for 15 min at 37°C with PI containing 50 μg/ml RNase (BD Pharmingen) followed by flow cytometric analysis.

**Statistical analysis**

All data were expressed as mean ± standard deviation (SD). Statistical significance was determined by t-test or one-way ANOVA for multiple comparisons. P < 0.05 was considered as statistically significant.

**Results**

**CFZ and vorinostat synergistically inhibited proliferation in Jurkat T-leukemia cells**

We first evaluated the inhibition of proliferation by CCK-8 assay using the leukemia cell line Jurkat cells. As shown in Fig. 1A, CFZ at low concentrations (≤8.0 nM) was minimally toxic in the Jurkat cells. But combination with very low concentrations of vorinostat (0.2, 0.3, or 0.4 μM) sharply increased its cytotoxicity when CFZ concentrations ≥ 6 nM. Similarly, ≤0.4 μM of vorinostat was marginally toxic in the Jurkat cells. But combination with low concentrations of CFZ (6, 7, or 8 nM) led to a marked cytotoxicity when vorinostat concentrations ≤0.2 μM (Fig. 1B). Median dose effect analysis of the interactions between various CFZ concentrations (2–10 nM) and fixed vorinostat (0.2, 0.3 or 0.4 μM) concentrations yielded combination index values substantially <1.0, denoting synergistic interactions (Fig. 1C).

CFZ and vorinostat synergistically induced apoptosis, caspase activation, and PARP cleavage in Jurkat T-leukemia cells

After incubating with CFZ (8 nM) or/and vorinostat (0.4 μM) for 24 or 48 h, apoptosis was examined by a flow cytometer. Combination treatment induced a significant increase in the percentage of apoptosis cells (early or late), which were 24.51% for 24 h and 46.90% for 48 h. However, the percentage of apoptosis cells (early or late) treated with CFZ (5.31% for 24 h, 5.86% for 48 h) or vorinostat (3.75% for 24 h, 5.88% for 48 h) alone was much lower than the combination treatment (Fig. 2A and Table 1).

In order to determine if caspase activation and PARP cleavage occur in CFZ/vorinostat-induced apoptosis, Jurkat cells were incubated with CFZ (8 nM) or/and vorinostat (0.4 μM) for 48 h. Western blot analysis showed that combination treatment significantly induced the activation of caspase-9 and -3 and the cleavage of PARP, but this activation was not clearly seen in the treatment with CFZ or vorinostat alone (Fig. 2B).

Combination of CFZ with vorinostat induce G2-M arrest in Jurkat T-leukemia cells

Jurkat cells were treated with CFZ (8 nM) or/and vorinostat (0.4 μM) for 48 h. As shown in Fig. 3, compared with the control, 8 nM CFZ increased the population of G2-M phase from 14.3% ± 3.4% to 42.5% ± 2.5% (n = 3, P < 0.05). However, combination treatment failed to induce more accumulation of cells in G2-M phase than CFZ alone (n = 3, P > 0.05). These results suggested that combination treatment induced G2-M arrest in the Jurkat cells, in which CFZ play a dominant role and seemingly have no synergistic interactions with vorinostat.

CFZ and vorinostat synergistically induced the loss of mitochondrial membrane potential and the release of cytochrome c

Jurkat cells were treated with CFZ (8 nM) or/and vorinostat (0.4 μM) for 24 h, stained with JC-1 dye, mitochondrial membrane potential was assessed by flow cytometric analysis. CFZ (8 nM) or vorinostat (0.4 μM) alone resulted in
little change in mitochondrial membrane potential, but combination treatment markedly decreased mitochondrial membrane potential (Fig. 4A).

To investigate whether the loss of mitochondrial membrane potential is followed by the released of cytochrome c, we detected the level of cytochrome c in cytosol of Jurkat cells treated with CFZ (8 nM) or/and vorinostat (0.4 μM) for 24 h. Western blot analysis revealed an increased level of cytochrome c in cytosol of the cells of combination treatment (Fig. 4B).

Increased ROS generation played a critical role in CFZ/vorinostat-induced apoptosis in Jurkat T-leukemia cells through the loss of mitochondrial membrane potential

Previous studies have reported that increased reactive oxygen species (ROS) levels play an important role in the mechanism of proteasome inhibitor and histone deacetylase inhibitors induced cell apoptosis [19,20]. Thus, firstly, we evaluated ROS production in the Jurkat cells treated with CFZ (8 nM) or/and vorinostat (0.4 μM) for 48 h. As shown
in Fig. 5A, single treatment with CFZ or vorinostat alone slightly increased the level of ROS; however, combination of CFZ with vorinostat markedly enhanced ROS generation over control (>2-fold). ROS scavengers N-acetylcysteine (NAC) blocked combination treatment-induced ROS generation. Secondly, we investigated whether increased ROS generation is involved in CFZ/vorinostat-induced apoptosis in Jurkat T-leukemia cells. When cells were pre-incubated with 10 mM ROS scavengers NAC for 3 h and then treated with the combination of 8 nM CFZ and 0.4 μM vorinostat for 48 h, ROS generation was blocked (Fig. 5A). Furthermore, apoptosis was attenuated (Fig. 5B). Increased ROS generation is associated with the loss of mitochondrial membrane potential [21]. Therefore, we investigated whether NAC attenuates CFZ/vorinostat-induced apoptosis through the prevention of the loss of mitochondrial membrane potential. When cells were pre-incubated with or without 10 mM ROS scavengers NAC for 3 h and then treated with the combination of 8 nM CFZ and 0.4 μM vorinostat for 48 h, the changes in mitochondrial membrane potential were examined. As shown in Fig. 5C, the loss of mitochondrial membrane potential was markedly rescued by pre-treatment with NAC. These findings indicated that increased ROS generation led to the loss of mitochondrial membrane potential, and then triggered apoptosis in the Jurkat T-leukemia cells.

Discussion

Previous studies have reported that proteasome inhibitor bortezomib and histone deacetylase inhibitors have synergistic interactions in a variety of malignancies [13,14]. Second-generation proteasome inhibitor CFZ has shown activity...
against bortezomib-resistant cells [7]. Therefore, the combination of CFZ with histone deacetylase inhibitors may have more potent synergistic interactions than cotreatment with bortezomib and histone deacetylase inhibitors. In addition, based on previous studies reporting that CFZ and vorinostat exert synergistic effects in other tumor types [17,18], we investigated whether the combination of histone deacetylase inhibitor vorinostat with the proteasome inhibitor CFZ would result in synergistic antitumor effects against a T-cell acute leukemia cell.

Our data showed that proteasome inhibitor CFZ and histone deacetylase inhibitor vorinostat resulted in a synergistic inhibition of cell proliferation and induction of cell apoptosis in the Jurkat T-leukemia cells. The synergy between CFZ and vorinostat in the Jurkat cells was accomplished by enhancing ROS generation, the loss of mitochondrial membrane potential, the increased release of cytochrome c, the activation of caspase-9 and -3, and the cleavage of PARP. Treatment of Jurkat cells with NAC substantially attenuated CFZ/vorinostat-mediated apoptosis in K562 cells. ROS generation plays a critical role in bortezomib/vorinostat-mediated apoptosis in K562 cells.

In addition, to further characterize the marked cytotoxicity of the combination treatment with CFZ and vorinostat, cell cycle analyses were performed. We found that treatment with CFZ alone resulted in a significant increase in G2-M phase. However, combination treatment failed to induce more accumulation of cells in G2-M phase than CFZ alone. The results suggested that CFZ play a dominant role and seemingly have no synergistic interactions with vorinostat in the induction of G2-M arrest in Jurkat cells, which is in agreement with Wang et al.’s study [15]. They reported that combination with valproic acid (VPA) and bortezomib induces a similar G2-M arrest in HL-60 and Mutz-1 cells compared with bortezomib alone. But the result was not consistent with the results of Dasmahapatra et al.’s [17]. Their data showed that CFZ plus vorinostat induce more pronounced cell cycle arrest in G2-M in DLBCL cells, compared with CFZ alone. On the one hand, the different results may be associated with the types of cells. On the other hand, we found that the concentration of vorinostat in their study was almost 4-fold than that in our study. It is possible that very low concentration vorinostat has not enough power to perform synergistic interaction with CFZ in cell cycle arrest.
Many cell signal molecules are involved in the regulation of the intrinsic/mitochondrial pathway, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [23], multiple intracellular signal-regulating kinase1/2 (ERK1/2) [22], serine/threonine kinase (AKT) [16], p38 mitogen-activated protein kinase (p38MAPK) [24], and c-jun N-terminal kinase (JNK) [17]. Further study on which signal molecules play a significant role in CFZ/vorinostat-mediated apoptosis in T-leukemia cells is now in progress in our laboratory. It was reported that apoptosis has two major signaling pathways, the extrinsic/death receptor pathway and the intrinsic/mitochondrial pathway [25].
Whether the extrinsic/death receptor pathway is also activated by CFZ and vorinostat in Jurkat T-leukemia cells is not clear and needs to be investigated.

In conclusion, our findings indicated that combination treatment with CFZ and vorinostat synergistically results in antiproliferative and proapoptotic effects against Jurkat T-leukemia cells, which is accomplished by enhancing ROS generation, the loss of mitochondrial membrane potential, the increased release of cytochrome c, the activation of caspase-9 and -3, and the cleavage of PARP. Although the exact mechanism remains unclear, our research provided a starting point to explore the combination of CFZ/vorinostat for the treatment of T-ALL.

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