

Cefepime, a fourth-generation cephalosporin, in complex with manganese, inhibits proteasome activity and induces the apoptosis of human breast cancer cells

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Abstract. Cefepime (FEP), which is a member of the fourth-generation cephalosporin class, has been extensively studied as a biochemical and antimicrobial reagent in recent years. Manganese (Mn) is important in the biochemical and physiological processes of many living organisms, and it is also high expressed in some tumor tissues. In the present study, we aimed to investigate the proteasome-inhibitory and anti-proliferative properties of 8 metal complexes (FEP-Cu, FEP-Zn, FEP-Co, FEP-Ni, FEP-Cd, FEP-Cr, FEP-Fe, FEP-Mn) in MDA-MB-231 human breast cancer cells. The FEP-Mn complex was found to be more potent in its ability to inhibit cell proliferation and proteasome activity than the other compounds tested. Moreover, the FEP-Mn complex inhibited proteasomal chymotrypsin-like (CT-like) activity and induced the apoptosis of breast cancer cells in a dose- and time-dependent manner. Furthermore, the MCF-10A cells were much less sensitive to the FEP complexes compared with the MDA-MB-231 breast cancer cells. These results demonstrated that the FEP-Mn(II) complex has the potential to act as a proteasome inhibitor and apoptosis inducer and therefore has possible future applications in cancer chemotherapy.

Introduction

Cefepime (FEP) is a member of the fourth-generation cephalosporin class with broad-spectrum activity against both Gram-positive and Gram-negative bacteria (1) that has been widely used for approved indications, such as febrile neutropenia, which is a major adverse effect in patients receiving cancer chemotherapy with or without radiotherapy (2-5). It is known that several drugs have better pharmacological properties when in the form of metal complexes (6). Thus, a number of cephalosporin metal complexes have been extensively studied as biochemical and antimicrobial reagents over the past decade (7-12). However, to date, only a few studies have investigated the antitumor activity of these metal complexes.

Manganese (Mn), an essential trace element required by the human body, has come to be recognized as being significant in many biological systems (13-15). A few papers have demonstrated that some Mn-based compounds possess superoxide dismutase (SOD)-like activity (16,17) and may be considered as potential anti-*Mycobacterium tuberculosis* agents (18). In addition, some Mn complexes have also exhibited antitumor activity against a variety of cancer cells. For example, Mn(II) complexes of 6,7-dicyanodipyridoquinoline and 2H-5-hydroxy-1,2,5-oxadiazolo[3,4-f][1,10-phenanthroline] have been found to exhibit antitumor activity due to DNA binding (19,20). The Mn(II) compound [(Adpa)Mn(Cl)(H₂O)] (Adpa = bis(2-pyridylmethyl)amino-2-propionic acid) was reported to target the mitochondria and to inhibit the proliferation of U251 human glioma cells (21). Moreover, Mn(II) complexes of quinoline derivatives have potential as attenuators of Ca²⁺ absorption in the mitochondria and can interfere with the metabolism of O₂ for cancer chemotherapy (22). The novel Mn(II) complexes of indolecarboxylic acids have been reported to display cytotoxicity and their antiproliferative activity against some cell lines (e.g., Jurkat derived from an acute T cell leukemia), is similar to that of cisplatin (23). However, although many studies have demonstrated that such Mn(II) complexes have potential for use as antitumor drugs, their mechanisms of action are not yet fully understood.

It is known that the ubiquitin-proteasome system (UPS) maintains protein homeostasis in human cells, and it is involved

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in highly regulated cellular processes, including cell cycle progression, proliferation, apoptosis and differentiation (24,25). It has been proven that the UPS is an important target in cancer treatment. The 20S proteasome, the main component of the UPS, possesses multiple peptidase activities, such as caspase-like or peptidyl-glutamyl peptide-hydrolyzing-like (PGPH-like) activity ($\beta 1$ subunit), trypsin like activity ($\beta 2$ subunit) and chymotrypsin-like (CT-like) activity ($\beta 5$ subunit) (26-28). However, only the inhibition of proteasomal CT-like activity has been shown to be closely associated with the induction of cancer cell death (29-31). In 2004, Daniel *et al* (32) found that mixtures of copper salts with the ligands (clioquinol, hydroxyquinoline and dithiocarbamate) inhibited the effect of the 20S proteasome *in vitro* and *in vivo*. Therefore, the inhibition of proteasomal CT-like activity is associated with the induction of cancer cell apoptosis (33,34) and is unquestionably linked to the presence of metal ions (35).

Previously, we found that certain types of copper(II) complexes, $[\text{Cu}(\text{C}_{10}\text{H}_8\text{O}_2\text{N})_2(\text{C}_{12}\text{H}_8\text{N}_2)]$ and $[\text{Cu}(\text{C}_{11}\text{H}_{10}\text{O}_2\text{N})_2(\text{C}_{12}\text{H}_8\text{N}_2)]$, cadmium(II) complexes, $[\text{Cd}_2(\text{C}_{12}\text{H}_{12}\text{O}_2\text{N})_4(\text{H}_2\text{O})_2] \cdot 2\text{H}_2\text{O}$, $[\text{Cd}_2(\text{C}_{11}\text{H}_{10}\text{O}_2\text{N})_4(\text{H}_2\text{O})_2] \cdot 2\text{H}_2\text{O}$ and $[\text{Cd}(\text{C}_7\text{H}_4\text{N}_2\text{O}_2)(\text{C}_8\text{H}_6\text{O}_2)_2] \cdot 2\text{H}_2\text{O}$, may serve as potent, selective proteasome inhibitors and apoptosis inducers in cultured human cancer cells (36,37). In this study, we investigated the effects of FEP-metal complexes on breast cancer cells. We found that only the FEP-Mn complex inhibited the CT-like activity of the proteasome in human breast cancer cell cultures and induced cancer cell death. Furthermore, the MDA-MB-231 breast cancer cells were more sensitive to the novel candidate FEP-Mn complex than normal cells. This suggests that the FEP-Mn complex has potential for use as a novel class of antitumor agents.

Materials and methods

Chemicals and reagents. FEP was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The chemical structure of FEP is illustrated in Fig. 1. Dimethylsulfoxide (DMSO) and the acetate $[\text{Cu}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, $\text{Co}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, $\text{Ni}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, $\text{Mn}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, $\text{Cd}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$] were all purchased from Aladdin Reagents Co. Ltd., (Shanghai, China). FEP and various metal salts were dissolved and formed new complexes [FEP-Cu (FEP1), FEP-Zn (FEP2), FEP-Co (FEP3), FEP-Ni (FEP4), FEP-Cr (FEP5), FEP-Cd (FEP6), FEP-Fe (FEP7), FEP-Mn (FEP8)] in DMSO to a final concentration of 50 mM and stored at 4°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Aleken Biologicals (Nash, TX, USA). The fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal CT-like activity) was obtained from Calbiochem (San Diego, CA, USA). Mouse monoclonal antibody against human poly(ADP-ribose) polymerase (PARP), mouse monoclonal antibodies against ubiquitin (P4D1) and I κ B- α (H-4), goat polyclonal antibody against β -actin (C-11) and all secondary antibodies were

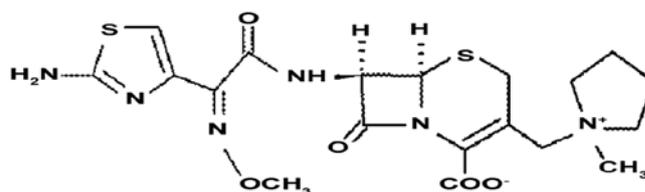


Figure 1. Chemical structure of cefepime (FEP).

purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and whole cell extract preparation. MDA-MB-231 human breast cancer cells were grown in DMEM/F-12 (1:1) supplemented with 10% FBS, 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin. MCF-10A cells (immortalized, but non-tumorigenic) were cultured in 1:1 DMEM/F-12 supplemented with 5% (v/v) horse serum, 0.029 mol/l sodium bicarbonate, 10 mM HEPES buffer solution, 100 U/ml of penicillin, 5 mg insulin, 10 μg of epidermal growth factor and 250 μg hydrocortisone. The cell lines were maintained at 37°C in an atmosphere containing 5% CO_2 . The whole cell extracts were prepared as previously described (38). Briefly, the cells were harvested, lysed and the supernatants were collected as whole-cell extracts which were used for western blot analysis.

Cell proliferation assays. The effect of each FEP-metal complex on cell proliferation was determined by MTT assay. MDA-MB-231 and MCF-10A cells were seeded in triplicate in a 96-well plate, grown to 70-80% confluence, and then treated with the indicated concentration of each FEP-metal complex. After 48 h of incubation at 37°C, the inhibition of cell proliferation was measured as previously described (38).

Inhibition of purified 20S proteasome activity by the FEP-Mn complex. The CT-like activity of the purified human 20S proteasome was measured as previously described (32). Briefly, 35 ng of purified 20S proteasome were incubated in 100 μl of assay buffer (20 mM Tris-HCl, pH 7.5) with or without various concentrations of the FEP-Mn complex and 20 μM of CT-like substrate Suc-LLVY-AMC (for the proteasomal CT-like activity) for 2 h at 37°C. Following incubation, proteasome CT-like activity was measured using the Wallac Victor 3 multilabel counter with an excitation filter of 365 nm and emission filter of 460 nm.

Analysis of proteasomal CT-like activity in whole-cell extracts. Whole-cell extracts (10 μg) of MDA-MB-231 breast cancer cells were incubated for 2 h at 37°C in 100 μl of assay buffer (20 mmol/l Tris-HCl, pH 7.5) with 20 $\mu\text{mol}/\text{l}$ fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal CT-like activity), followed by measurement of AMC group release, as described above.

Western blot analysis. Breast cancer cells were treated with the different complexes as indicated in the figure legends. Cells treated with DMSO served as the controls. After 24 h of treatment, the cells were harvested and lysed. Protein (40 μg) from whole-cell extracts was separated by sodium dodecyl sulfate

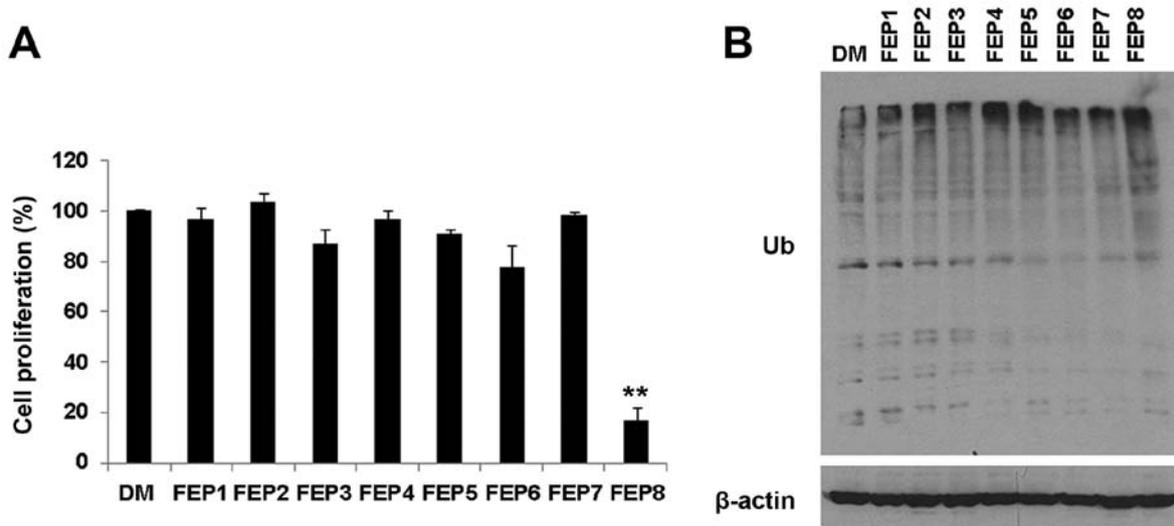


Figure 2. Comparison of cell proliferation and proteasome inhibition in MDA-MB-231 cells treated with the cefepime and metal complexes (FEP1-FEP8). MDA-MB-231 cells were treated with 40 μ M of each indicated compound for 24 h. (A) MTT assay was performed to assess cell proliferation. **P<0.01, compared to other treatments. Bars indicate the means \pm SD of 3 independent experiments. (B) Western blot analysis of polyubiquitinated proteins was performed. β -actin was used as a loading control.

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Western blot analysis was performed using specific antibodies to ubiquitin, I κ B- α , PARP, and β -actin, followed by visualization with an enhanced chemiluminescence reagent, as previously described (39).

Analysis of cell morphology. Changes in cell morphology were observed using a Zeiss Axiovert 25 phase contrast microscope (Zeiss, Oberkochen, Germany). Rounded and detached cells were considered apoptotic.

Statistical analysis. Statistical analysis was performed using Microsoft Excel software. Differences between groups were analyzed using the Student's t-test. All data are presented as the means \pm SD.

Results

FEP forms new complexes with different metal salts in solution. Several previous studies have reported that many complexes, such as clioquinol-copper (40), the ternary complexes, indole-3-acetic acid-copper-phenanthroline (phen) and indole-3-propionic-copper-phen (36), taurine and L-glutamine Schiff base copper complexes (41,42), zinc, nickel, cadmium complexes and so on, are capable of inhibiting cell proliferation and proteasome activity, and inducing the apoptosis of human cancer cells (37,43,44). However, to the best of our knowledge, the effects of Mn(II) complexes on cancer cells have not been reported to date. In the current study, FEP was mixed with various metal salts, dissolved in DMSO at a concentration of 50 mM, in a 1:1 molar ratio. The reaction of FEP with the different metal salts resulted in a marked change in color, which indicated that a chemical reaction had occurred and the complexes had formed (data not shown). In this study, we focused on the FEP-metal complex solution and investigated the effects of this complex on human breast cancer cells.

Antiproliferative and proteasome-inhibitory effects of FEP1-FEP8 in MDA-MB-231 breast cancer cells. For this portion of the study, the FEP1-FEP8 complexes were investigated for their growth inhibitory effects on MDA-MB-231 breast cancer cells at the concentration of at 40 μ M of each compound for 24 h. Cells treated with DMSO were used as a control. Of note, the most effective compound was found to be FEP8, the FEP-Mn complex, which induced the greatest decrease in the proliferation of MDA-MB-231 cells, causing a >83% growth inhibition after 24 h of treatment (Fig. 2A). However, all the other FEP-metal complexes caused little or no growth inhibition of MDA-MB-231 breast cancer cells (Fig. 2A).

We then measured the ability of complexes FEP1-FEP8 to inhibit proteasome activity. The MDA-MB-231 cells were treated with 40 μ M of each compound for 24 h and the effect on accumulated ubiquitinated proteins was assessed. The results of western blot analysis confirmed that the FEP-Mn complex was a potent proteasome inhibitor. The accumulation of ubiquitinated proteins was observed in the cells treated with FEP8, but not in the cells treated with the other complexes (Fig. 2B). Thus, our results suggest that FEP8 is more potent in its ability to inhibit cell proliferation and proteasome activity than the other compounds tested (FEP1-FEP7).

In our previous studies, we demonstrated that a number of organic copper- and cadmium-based complexes were capable of inhibiting the tumor cell proteasome and thus, cell proliferation, thereby inhibiting cancer cell growth (36,37). It was of interest to determine whether this was also an effect of the Mn complex.

Inhibition of purified 20S proteasome activity by FEP8. To investigate the effects of the Mn complex, we also carried out *in vitro* assays using the purified 20S proteasome, which was treated with various concentrations of FEP8 and with DMSO as a control. The results revealed that this Mn complex is capable of inhibiting the CT-like activity of the purified

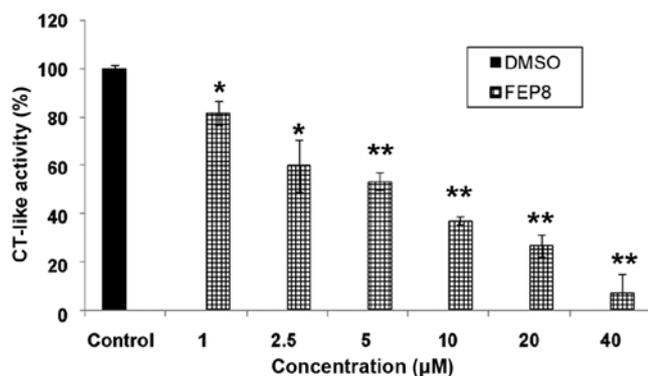


Figure 3. Purified 20S proteasome inhibition by the cefepime and manganese complex (FEP8). Purified human 20S proteasome (35 ng) was incubated with dimethylsulfoxide (DMSO) or various concentrations of FEP8 for 2 h, and a proteasomal chymotrypsin (CT)-like activity assay was performed. * $P < 0.05$ and ** $P < 0.01$, compared to control. Bars indicate the means \pm SD of 3 independent experiments.

20S proteasome (Fig. 3). This indicated that the FEP-Mn complex targeted the 20S proteasomal catalytic $\beta 5$ subunit. This is consistent with the results of our previous studies (36,37), which demonstrated that copper and cadmium complexes have the potential to serve as potent, selective proteasome inhibitors.

Inhibition of CT-like activity and induction of apoptosis by FEP8 in MDA-MB-231 breast cancer cells. In the previous section, we have discussed the inhibition of proteasomal CT-like activity under cell-free conditions. We then sought to determine whether this effect is dose dependent in cancer cells. The MDA-MB-231 cells were treated with FEP8 at concentrations of 20, 30, 40 and 50 μ M. Cells treated with DMSO were used as a vehicle control. After 24 h of treatment, the cells were

collected and cell extracts were prepared for the determination of the extent of proteasome inhibition. The inhibition of cellular proteasome activity was measured as the decreased levels of CT-like activity and the accumulation of ubiquitinated proteins, as previously described (45).

The results revealed that FEP8 inhibited the proteasomal CT-like activity in a dose-dependent manner by approximately 40% at 20 μ M, 70% at 30 μ M, 82% at 40 μ M and 92% at 50 μ M (Fig. 4A). Consistently, the dose-dependent accumulation of ubiquitinated proteins was also observed in the MDA-MB-231 breast cancer cells treated with FEP8 (Fig. 4B). It has been reported that the inhibition of the proteasomal CT-like activity is associated with the apoptosis of cancer cells (34).

In the same experiment, we then evaluated the apoptosis-inducing effect of FEP8 in breast cancer cells. The cleaved p85 fragment of PARP was observed at an FEP8 concentration of 50 μ M (Fig. 4B), indicating the induction of cell apoptosis. To further investigate the apoptosis-inducing effect of FEP8, we examined changes in cell morphology. Treatment with FEP8 induced the rounding and shrinkage of the MDA-MB-231 cells (Fig. 4C) in a concentration-dependent manner. These results demonstrated that FEP8 possesses the ability to inhibit proteasome activity and is able of inducing the apoptosis of MDA-MB-231 human breast cancer cells in a dose-dependent manner.

Time-dependent effect of FEP8 on proteasome inhibition and induction of apoptosis in MDA-MB-231 breast cancer cells. In order to examine the time-dependent effect of FEP8-induced proteasome inhibition, the MDA-MB-231 cells were treated with 20 μ M of FEP8 for 3, 9, 24 or 48 h (DMSO-treated cells were used as a vehicle control), followed by the measurement of proteasome activity (Fig. 5). Proteasomal CT-like activity

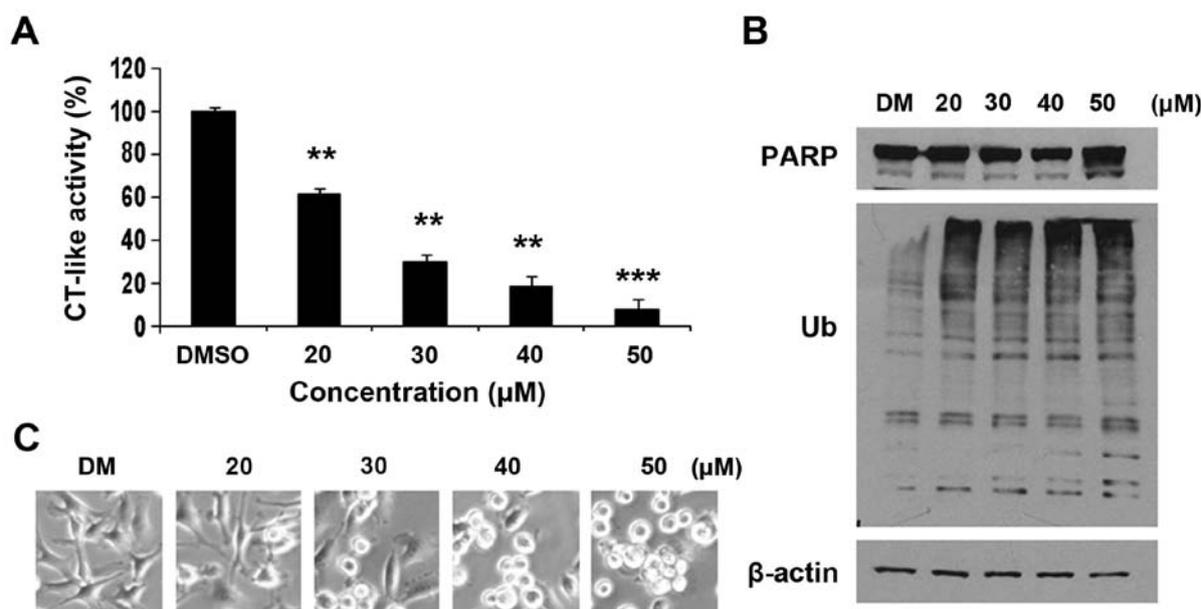


Figure 4. Effect of various concentrations of the cefepime plus manganese complex (FEP8) on MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated with dimethylsulfoxide (DMSO) (as a control) or various concentrations of FEP8 for 24 h. (A) Chymotrypsin (CT)-like activity assay. ** $P < 0.01$ and *** $P < 0.001$, compared to control. Bars indicate the means \pm SD of 3 independent experiments. (B) Western blot analysis of polyubiquitinated proteins and poly(ADP-ribose) polymerase (PARP), and (C) analysis of changes in cell morphology was performed.

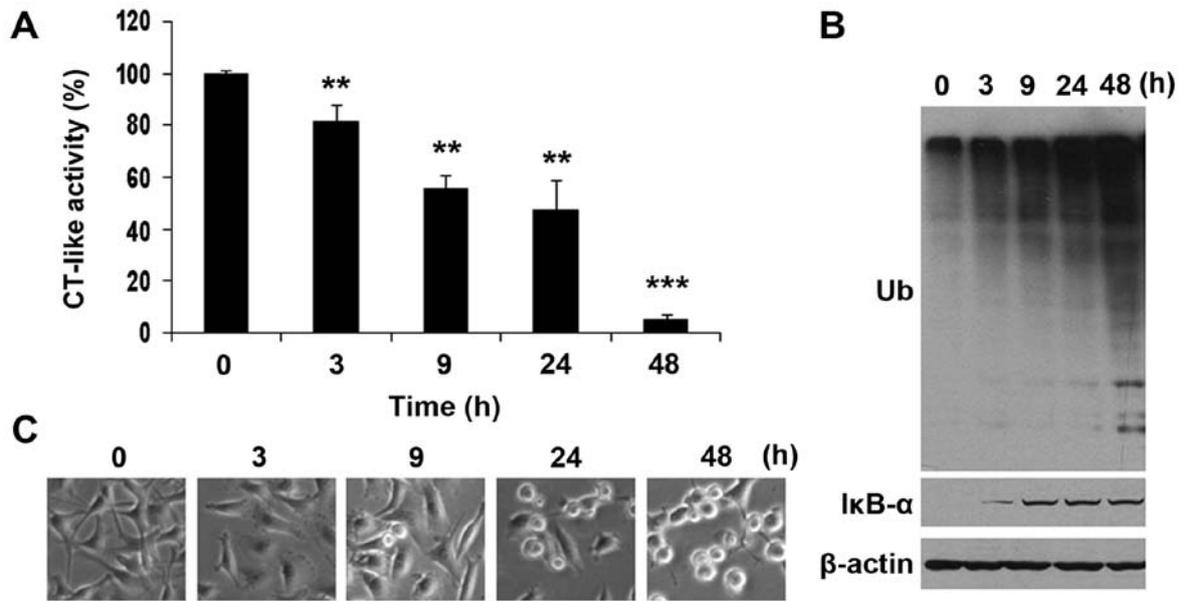


Figure 5. Time-dependent effect of proteasome inhibition and the induction of apoptosis by the cefepime plus manganese complex (FEP8) in MDA-MB-231 cells. MDA-MB-231 cells were exposed to 20 μ M of FEP8 for the indicated periods of time. (A) Proteasomal chymotrypsin (CT)-like activity assay. ** $P < 0.01$ and *** $P < 0.001$, compared to control. Bars indicate the means \pm SD of 3 independent experiments. (B) Western blot assay (ubiquitin, I κ B- α and β -actin), and (C) analysis of morphological changes associated with apoptotic cells were performed. The appearance of rounded and detached cells, indicative of apoptosis, gradually increased as time progressed.

was inhibited by FEP8 in a time-dependent manner, as early as 3 h, with ~20% inhibition (Fig. 5A). The CT-like activity was further decreased at subsequent time-points, with 45, 55 or 95% inhibition observed after 9, 24 and 48 h, respectively (Fig. 5A). Furthermore, the inhibition of proteasomal CT-like activity was associated with the gradual accumulation of ubiquitinated proteins, starting at 3 h and peaking at 48 h (Fig. 5B). Additionally, higher levels of the proteasomal target protein, I κ B- α , were also observed at 3 h post-treatment and later time-points (Fig. 5B). In the same time-dependent experiment, abnormal morphological changes were not detected until 9 h post-treatment with FEP8. Moreover, the appearance of rounded and detached cells, indicative of apoptosis, gradually increased as time progressed (Fig. 5C). These results clearly indicate that FEP8 induces proteasome inhibition, followed by the induction of apoptosis in breast cancer cells. Therefore, the apoptosis induced by FEP8 is a consequence of proteasome inhibition.

Less toxic effect of FEP8 on immortalized, non-tumorigenic MCF-10A breast cells compared with MDA-MB-231 breast cancer cells. It is imperative for effective anticancer drugs to have the ability to distinguish between normal and cancer cells (46-48). Thus, to investigate whether FEP8 exhibited selectivity toward cancer cells, but not normal cells, we used the MDA-MB-231 breast cancer cells and normal immortalized MCF-10A human breast cells. The two cell lines were treated with various concentrations of FEP8 for 24 h, and with DMSO as a control, followed by the measurement of cell proliferation and the observation of changes in cell morphology. When FEP8 was used at a concentration of 10 μ M, no inhibition of cell proliferation was observed in the MCF-10A cells, whereas ~20% inhibition of proliferation was observed in

the MDA-MB-231 cells (Fig. 6A). FEP8 exhibited markedly different effects on cell proliferation when the two cell lines were treated with FEP8 at concentrations ranging from 20 to 50 μ M, causing 56, 76, 87 and 95% growth inhibition of MDA-MB-231 breast cancer cells and 5, 14, 25 and 30% inhibition of non-tumorigenic MCF-10A cells, respectively (Fig. 6A). In this regard, it may be concluded that FEP8 is a potent inhibitor of MDA-MB-231 cell proliferation, but that it is less toxic to non-tumorigenic MCF-10A breast cells, thus rendering our novel Mn complexes more favorable for further preclinical studies.

Additionally, under the same conditions, morphological changes were meaningful in determining whether FEP8 induces less apoptosis in normal MCF-10A cells compared with MDA-MB-231 breast cancer cells (Fig. 6B). Shrunken and rounded MDA-MB-231 cells were observed following treatment with various concentrations of FEP8, whereas little or nearly no morphological changes were observed in the MCF-10A cells (Fig. 6B), which was consistent with the results of MTT assay. Taken together, our results suggest that FEP8 inhibits cell proliferation and induces apoptosis selectively in human cancer cells and exerts less toxic effects on normal immortalized breast cells.

Discussion

Since the discovery of the antitumor activity of cisplatin and its analogs, novel metal complexes have been studied extensively in chemotherapy (49). Mn is important in the biochemical and physiological processes of many living organisms (50). Mn is also highly expressed in some tumor tissues (51). Based on the biological function of Mn, many Mn complexes have been reported to exert anticancer activity in some cancer cell

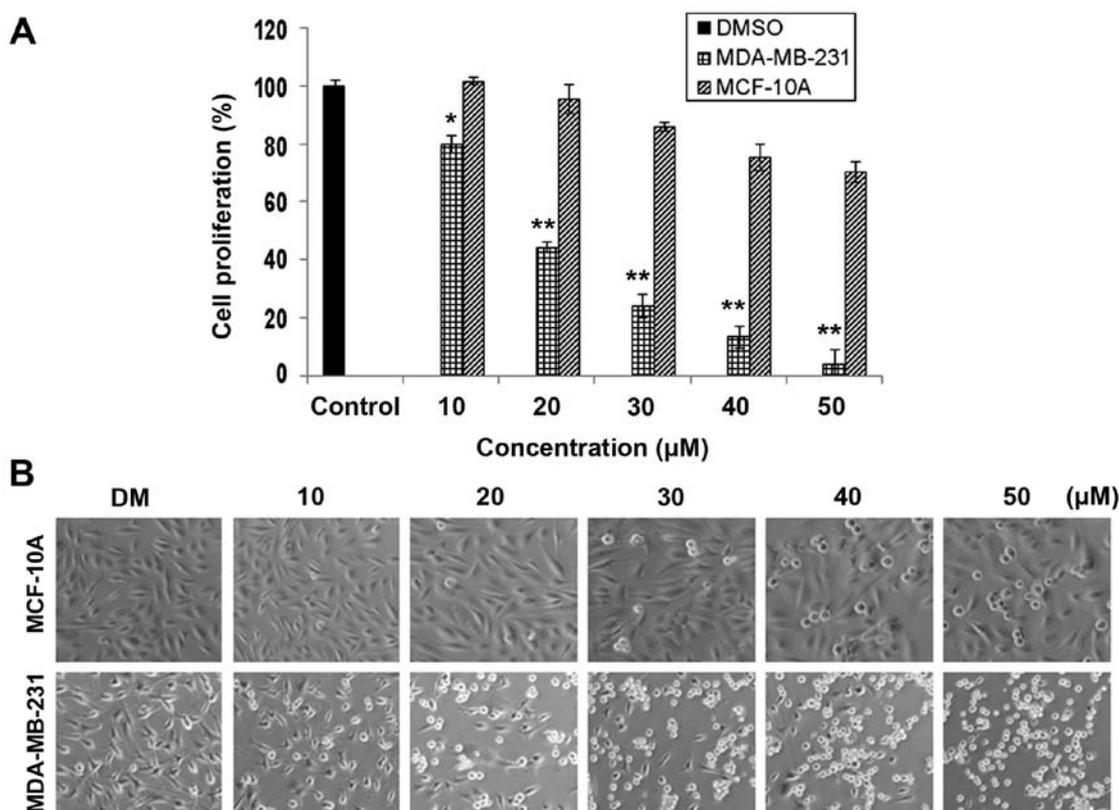


Figure 6. Differential effects of the cefepime plus manganese complex (FEP8) on MDA-MB-231 breast cancer cells and non-tumorigenic MCF-10A breast cells. MDA-MB-231 and MCF-10A Cells were treated with various concentrations of FEP8 or dimethylsulfoxide (DMSO) for 24 h. (A) An MTT assay was performed. * $P < 0.05$ and ** $P < 0.01$, compared to control. Bars indicate the means \pm SD of 3 independent experiments. (B) Morphological changes were observed. Shrunken and rounded MDA-MB-231 cells were observed following treatment with various concentrations of FEP8, whereas little or nearly no morphological changes were observed in the MCF-10A cells.

lines (19-22,52,53). Therefore, combining Mn(II) with several functional molecules has aroused considerable interest for the development of potent anticancer drugs.

We have previously reported that several organic copper and cadmium complexes possess the ability to inhibit proteasome activity and induce apoptosis in human cancer cells (36,37). However, in the present study, we found that only the FEP-Mn complex (FEP8) had the ability to inhibit cell proliferation and proteasome activity (Fig. 2). Therefore, FEP8 was selected for further study. Further experiments indicated that FEP8 inhibited the CT-like activity of the purified 20S proteasome (Fig. 3). Moreover, the inhibition of cellular proteasome activity was measured as the decreased levels of CT-like activity and the accumulation of ubiquitinated proteins (Fig. 4). With respect to proteasome-inhibitory activity, this FEP-Mn complex exhibited high activity toward not only the purified 20S proteasome, but also the proteasome from whole-cell extracts. Cellular morphological changes (shrinkage and rounding) were observed in a concentration-dependent manner (Fig. 4C). In line with these observations, FEP8 also induced the cleavage of PARP to form the p85 fragment (Fig. 4B), indicative of the induction of cell apoptosis. Thus, these results suggest that FEP8 has the potential for development as a novel anticancer agent.

The mechanism of action for the Mn complexes is suggested as follows: i) a Mn complex interacts with DNA and causes DNA damage, ultimately blocking the division of cancer cells and resulting in cell death (19,20); ii) a Mn(II) complex attenuates

the absorption of calcium(II) in the mitochondria and interferes with the metabolite of O_2 formed by H_2O_2 or ROS involved signaling, causing the apoptosis of cancer cells (21,22,52); iii) a Mn complex reduces the number of tumor-associated myeloid-derived suppressor cells, leading to the modulation of the immunosuppressive tumor microenvironment (TME) (53). However, our investigation revealed that the Mn complex (FEP8) inhibited 20S proteasome activity and directly inhibited CT-like activity, which is primarily associated with the $\beta 5$ active site (26). In addition, it has been reported that the decreased proteasomal CT-like activity is associated with the loss of cancer cell availability (35). In this study, it is interesting to note that the Mn complex inhibited proteasomal CT-like activity as early as 3 h (Fig. 5A), which was consistent with our results demonstrating the accumulation of ubiquitinated proteins and the target protein, $I\kappa B-\alpha$ (Fig. 5B). In addition, changes to cell morphology occurred 9 h after treatment began (Fig. 5C). In summary, our findings indicate that FEP8 has the potential to act as a specific inhibitor of proteasomal CT-like activity and thus to induce cell apoptosis.

A significant challenge in the development of novel anticancer drugs is the safety of metal compounds. Therefore, in this study, we examined whether FEP8 selectively inhibits cell proliferation and induces the apoptosis of MDA-MB-231 breast cancer cells, but not that of normal human MCF-10A breast cells. Indeed, our results demonstrated that the FEP-Mn complex had little effect on MCF-10A cells in contrast to

its effect on MDA-MB-231 breast cancer cells (Fig. 6). We noted that the FEP-Mn complex is a potent inhibitor of cell proliferation, which is specific to MDA-MB-231 breast cancer cells (Fig. 6A). Also noted was the fact that the MDA-MB-231 cells became detached and rounded when treated with the FEP-Mn complex and almost no toxicity was observed in the MCF-10A cells (Fig. 6B).

In conclusion, the data presented herein suggest that the FEP-Mn complex is a ligand could act as a potent proteasome inhibitor and inducer of apoptosis, and may be a potential anti-cancer agent targeting breast cancer cells, rather than normal cells. Therefore, this FEP-Mn complex has great potential for development into a drug for cancer treatment. However, further biological analysis and preclinical testing are warranted using animal tumor models.

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