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Sodium diethyldithiocarbamate, an AIDS progression inhibitor and a copper-binding compound, has proteasome-inhibitory and apoptosis-inducing activities in cancer cells

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Abstract. Diethyldithiocarbamate (DDTC) is a member of the dithiocarbamate family and a potent copper-chelating agent. DDTC was used in a clinical trial for patients with HIV-1 infection and showed a significant delay in progression to AIDS. In this study, we investigated the effects of DDTC-copper complex in human prostate and breast cancer cells. We found that DDTC was capable of binding copper and forming a new complex that potently inhibited the proteasomal chemotrypsin-like activity, decreased expression of androgen receptor (AR), estrogen receptor (ER) α and ERß proteins, and induced apoptosis in both prostate and breast cancer cells. Our data support the concept of using accumulated copper in cancer cells and tissues as a novel target for chemotherapy. This study provides a mechanistic interpretation for utilization of copper chelators in cancer treatment.

Introduction

The trace element copper is vital to the healthy functioning of organisms. Copper is used in a multitude of cellular activities (1,2). It is known that copper is involved in the development of cancers and important for both carcinogenesis and angiogenesis that plays a key role in tumor formation (3,4). Copper appears to act as an essential cofactor for several angiogenic growth factors (5). High levels of copper have been found in many types of human cancers (6-8). It has also been shown that the content of copper in serum and tissue of breast cancer patients was much higher than in benign breast tissues (9,10). Therefore, some metal chelators such as clioquinol have been shown to have tumor cell growth-inhibitory effects (11).

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Dithiocarbamates are sulfur-based chelators (12). Dithiocarbamates have numerous applications in chemical, agriculture, and pharmaceutical industries because of their metal-binding and antioxidant properties (13). Dithiocarbamates are a family of compounds with diverse biological activities, suggesting that it may have multiple, clinical uses. As a potent copper chelating agent, dithiocarbamates are widely used as therapeutic agents for treating alcoholism (14), metal poisoning (15), and AIDS (16-18). Diethyldithiocarbamate (DDTC) (Fig. 1A) is one of the dithiocarbamate compounds, a synthetic immunomodulator belonging to class I thymomimetic drugs, which could accelerate maturation and differentiation of prothymocytes and modulate the functions of mature T lymphocytes (19). DDTC has previously been observed to promote T-cell maturation in animal models and to reduce lymphadenopathy and improve survival in a murine AIDS model (16,17).

The ubiquitin/proteasome system plays an important role in degradation of cellular proteins. It possesses at least three distinct activities: chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues), and caspase-like (cleavage after acidic residues) (20). Inhibition of the proteasomal chymotrypsin-like activity has been found to be associated with induction of tumor cell apoptosis (21,22).

Apoptosis represents active, programmed cell death. It is a very important intracellular regulatory process that eliminates abnormal and potentially harmful cells. Based on morphological changes, apoptosis is characterized by plasma membrane blabbing, condensation, and fragmentation of cells and nuclei, as well as extensive degradation of chromosomal DNA into nucleosomal units. The deregulation of apoptotic mechanism contributes to a variety of diseases, such as cancer.

Previous studies in our laboratory demonstrated that certain types of copper-containing compounds, such as 8-hydroxyquinoline-copper and pyrrolidine dithiocarbamate-copper, are potent proteasome inhibitors and apoptosis inducers in cultured human cancer cells (11,23). In this study, we investigated the effects of DDTC-copper complex in human prostate and breast cancer cells. We found that DDTC is capable of binding copper and forming a new complex. The DDTC-copper complex can inhibit proteasome activity and induce apoptosis in both prostate and breast cancer cells, associated with decreased expression of AR and ER proteins. These studies

suggest the potential use of DDTC in the treatment of human cancer containing high levels of copper.

Materials and methods

Chemicals and reagents. Sodium diethyldithiocarbamate trihydrate, $CuCl_2$, dimethylsulfoxide (DMSO), and bisbenzimide Hoechst #33258 stain were purchased from Sigma-Aldrich. RPMI-1640 medium, fetal bovine serum, penicillin and streptomycin were purchased from Invitrogen. Minimum Essential Eagle Medium was purchased from ATCC. Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was obtained from Calbiochem. Mouse monoclonal antibody to human poly(ADP-ribose) Polymerase (PARP) was from Biomol International LP. Mouse monoclonal antibody to human androgen receptor (AR), mouse monoclonal antibody to human ubiquitin, rabbit monoclonal antibodies to human estrogen receptor α (ERα) and ß (ERß), goat polyclonal antibody to human actin were from Santa Cruz Biotechnology Inc. Caspase-3/-7 inhibitor was purchased from Bachem. Enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences.

Cell culture and cell lysates preparation. Prostate (LNCaP and C4-2B) and breast (MDA-MB-231) cancer cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Breast cancer MCF-7 cells were cultured in Minimum Essential Eagle Medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1% insulin. All cell lines were maintained at 37˚C in a humidified incubator with an atmosphere of 5% CO_2. Whole cell extracts were prepared as described previously (11,23). Briefly, cells were harvested, washed with PBS twice, and homogenized in a lysis buffer (50 mM Tris-HCI, pH 8.0, 150 mM NaCI, 0.5% NP40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4˚C. Afterwards, the lysates were centrifuged at 12,000 g for 15 min, and the supernatants were collected as whole cell extracts.

Color change and reaction of DDTC-copper complex. DDTC and $CuCl_2$ were dissolved in DMSO to a final concentration of 20 mM. Then both solutions were mixed in a 1:1 ratio and qualitatively examined for color change.

Cellular and nuclear morphology analysis. A Zeiss Axiovert 25 microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology with Hoechst staining. For fluorescent nuclear morphology analysis, Hoechst stain was used as follows. Cells, either attached in plates or collected as a detached fraction, were washed with ice-cold PBS. Cells were then fixed in 70% ethanol for 1 h and afterwards washed with ice-cold PBS. Cells were stained with 50 μM Hoechst and kept in dark at 4˚C for 30 min. Nuclei were visualized using fluorescence microscopy. Punctuated and bright staining, or granular and bright staining nuclei were considered apoptotic.

Western blot analysis. Prostate and breast cancer cells were treated with 10 μM copper, 10 μM DDTC, or DDTC-copper complex at 1-10 μM as indicated. Control cells were treated with DMSO. After 24 h of treatment, cells were harvested and lysed. Cell lysates (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed using antibodies to ubiquitin, PARP, AR, ERα, ERß or actin, followed by visualization using the enhanced chemiluminescence (ECL) kit.

Proteasomal chymotrypsin-like activity assay. Whole cell extract (10 μg) from prostate or breast cancer cells treated as indicated was incubated for 60 min at 37˚C in 100 μl of assay buffer (50 mM Tris-HCl, pH 7.5) with 20 μM of fluorogenic substrate for the proteasomal chymotrypsin-like activity. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor 3 multilabel counter with an excitation filter of 380 nm and an emission filter of 460 nm. Changes in fluorescence were calculated against non-treated controls and plotted with statistical analysis using Microsoft Excel™ software.

Statistical analysis. Statistical analysis was performed with Microsoft Excel software.

Results

DDTC and copper interact in solutions and form a new complex. The concept to be tested is that the high level of endogenous copper in tumors can be used as a target for cancer therapy. To exploit the potential use of DDTC as a copper-binding compound, we need to confirm the interaction of DDTC and copper in solutions. To do so, 20 mM of DDTC (colorless, Fig. 1B) was mixed with 20 mM of copper chloride (light green) at 1:1 molar ratio. The reaction of DDTC with copper resulted in a dramatic color change to dark brown (Fig. 1B), which indicated that a chemical reaction had occurred and a DDTC-copper complex formed. This result suggests that DDTC as a strong copper chelator should be capable of interacting with endogenous tumor copper.

The proteasome-inhibitory effect of the DDTC-copper complex in prostate cancer cells. Human prostate cancer LNCaP cells (androgen-dependent) contain a wild-type AR protein (24), while C4-2B cells (androgen-independent) express a constitutively activated mutant AR protein (25). To examine whether the *in vitro* formed DDTC-copper complex (Fig. 1B) was capable of inhibiting cellular proteasome activity, both LNCaP and C4-2B cell lines were treated with 10 μM copper alone, 10 μM DDTC alone, or the DDTC-copper complex at 1-10 μM. Cells treated with DMSO were used as control. After 24 h of treatment, the cells were collected and cell extracts were prepared for determination of proteasome inhibition. Inhibition of cellular proteasome activity was measured as the decreased levels of the chymotrypsin-like activity and accumulation of ubiquitinated proteins. The DDTC-copper complex significantly inhibited the proteasomal chymotrypsin-like activity in both LNCaP and C4-2B cell lines in a concentration-dependent manner (Fig. 2A and B). At a concentration of 5 μM, DDTC-copper caused 70 and 90% inhibition of the proteasomal chymotrypsin-like activity in LNCaP and C4-2B cell lines, respectively, when compared

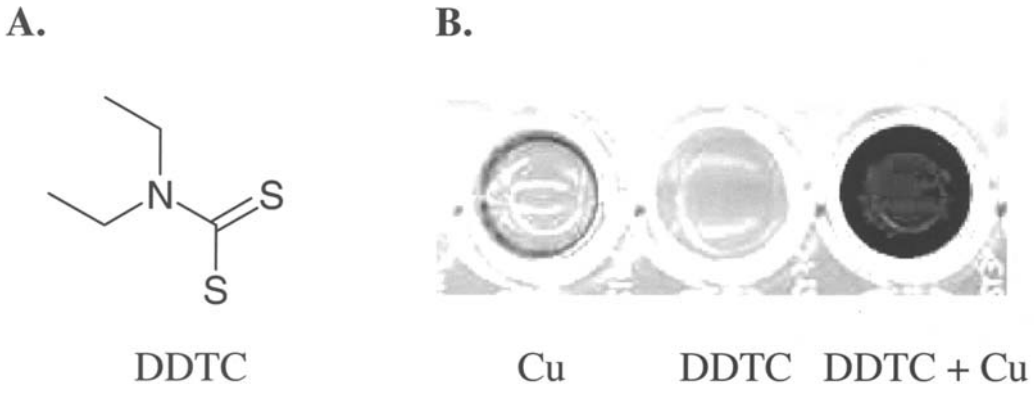

Figure 1. Formation of DDTC-copper complex. (A) Chemical structure of DDTC. (B) DDTC (20 mM, colorless) is mixed in a 1:1 molar ratio with 20 mM $CuCl_2$ (light color) to form DDTC-copper complex as indicated by color change (dark color).

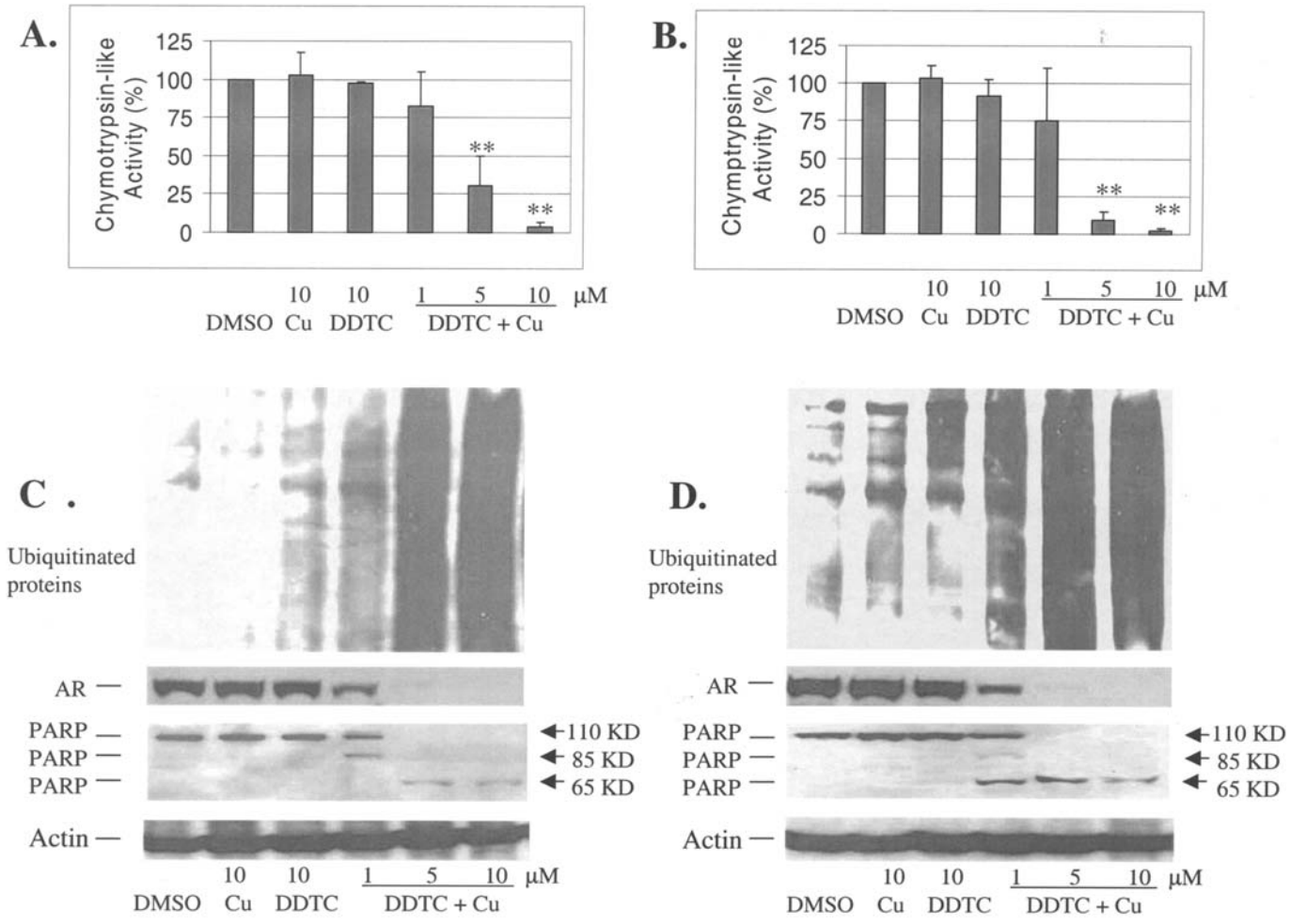

Figure 2. The effects of proteasome inhibition and apoptosis induction of DDTC-copper complex in prostate cancer cells. (A) The proteasomal chymotrypsin-like activity significantly decreased in LNCaP cells treated with 5 or 10 μM of DDTC-copper complex compared with control cells ($^{**}P \leq 0.01$). (B) Chymotrypsin-like activity significantly decreased in C4-2B cells treated with 5 or 10 μM of DDTC-copper complex compared with control ($^{**}P \leq 0.01$). (C) Western blot analysis for accumulated ubiquitinated proteins, AR expression, PARP cleavage, and actin expression in LNCaP cells. (D) Western blot analysis for accumulated ubiquitinated proteins, AR expression, PARP cleavage, and actin expression in C4-2B cells.

with the control cells (Fig. 2A and B). In contrast, copper at 10 μM had no inhibitory effect, while DDTC at 10 μM caused <10% proteasomal inhibition (Fig. 2A and B).

In order to determine the accumulation of ubiquitinated proteins, the whole cell lysates were subjected to SDS-PAGE and analyzed with anti-ubiquitin antibody. The accumulated ubiquitinated proteins were observed in cells treated with various concentrations of DDTC-copper complex in both LNCaP and C4-2B cell lines (Fig. 2C and D). Neither copper alone nor DDTC alone resulted in these effects. These results indicated that DDTC-copper complex inhibited proteasome activity in both prostate cancer cell lines.

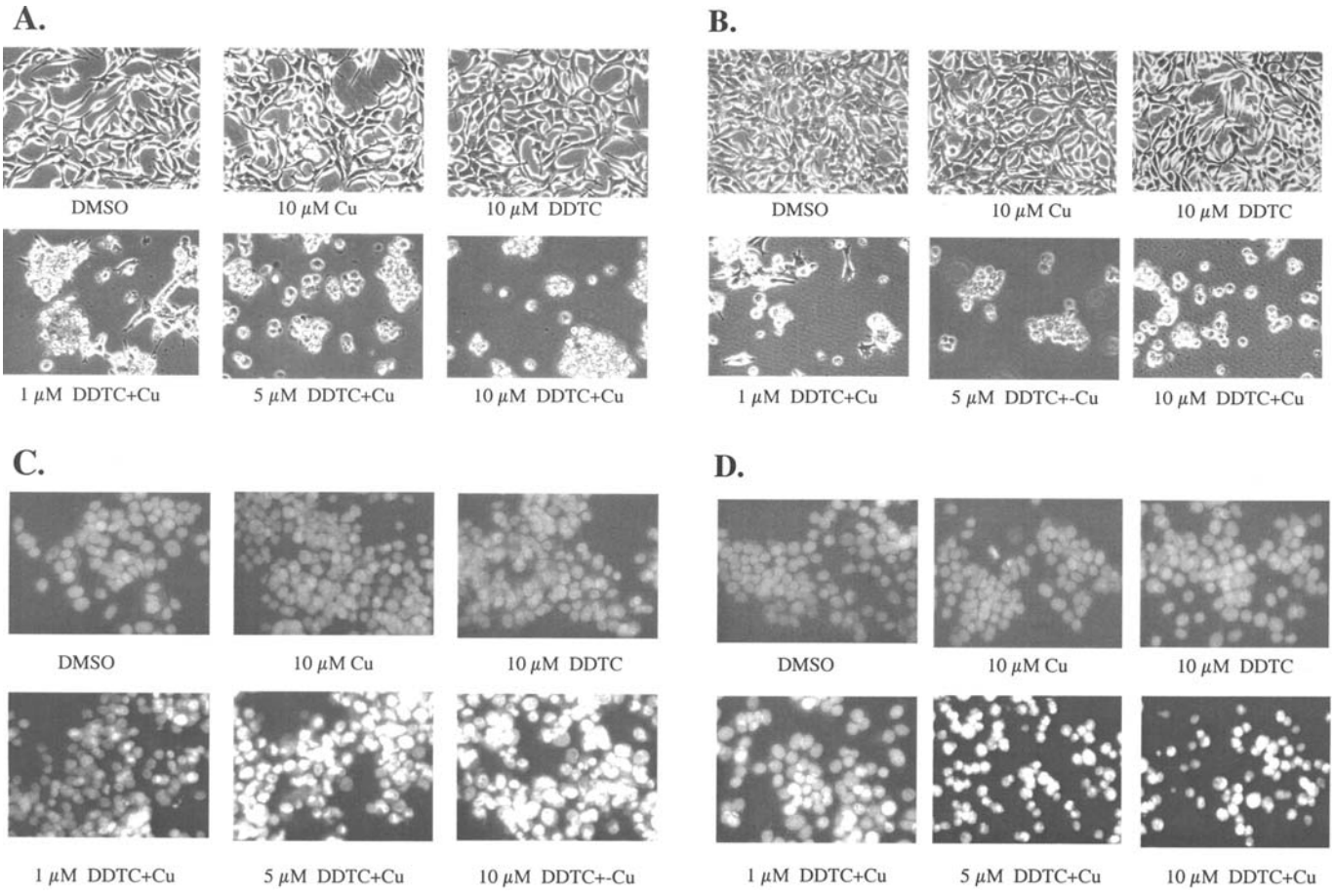

Figure 3. The cellular and nuclear morphological changes of prostate cancer cells treated with DDTC-copper complex. (A) Cellular spherical and detached changes are shown in LNCaP cells treated with various concentrations of DDTC-copper. (B) Cellular spherical and detached changes in C4-2B cells treated with DDTC-copper. (C) Punctuated, or granular, and bright nuclei indicating apoptotic nuclei were observed in LNCaP cells treated with various concentrations of DDTC-copper complex. (D) Apoptotic nuclei in C4-2B cells treated with DDTC-copper complex.

The effect of DDTC-copper complex on induction of apoptosis in prostate cancer cells. To determine whether the DDTC-copper complex could affect levels of AR protein in LNCaP and C4-2B cell lines, whole cell lysates prepared from the above treated prostate cancer cells were subjected to SDS-PAGE and analyzed with anti-AR antibody. The AR protein level decreased in cells treated with various concentrations of DDTC-copper (Fig. 2C and D). However, neither DDTC nor copper alone had an effect on AR decrease in these cell lines.

We then evaluated the apoptosis-inducing potency of DDTC-copper in the prostate cancer cells. Aliquots of the samples of the treated prostate cancer cells were subjected to Western blot analysis with anti-PARP antibody. Cleavage of PARP was observed in both LNCaP and C4-2B cells treated with DDTC-copper complex (Fig. 2C and D), indicating apoptosis induction. Furthermore, the PARP cleavage was DDTC-copper complex concentration-dependent (Fig. 2C and D). In contrast, neither DDTC nor copper alone had similar effects (Fig. 2C and D).

To further study the apoptotic cell death-inducing effect of the DDTC-copper complex, we measured cellular and nuclear morphological changes. Similarly, LNCaP and C4-2B cells were treated with 10 μM copper, 10 μM DDTC, 1-10 μM DDTC-copper complex, or DMSO for 24 h. Cellular morphology changes were visualized by phase contrast imaging. Cellular spherical and detached changes indicating apoptotic cell death were observed in both LNCaP and C4-2B cells treated with various concentrations of DDTC-copper complex (Fig. 3A and B). Such cellular morphological change was not observed in control cells, or cells treated with either copper or DDTC alone (Fig. 3A and B).

To determine the presence of apoptotic nuclei, aliquots of the treated cells were stained with Hoechst dye. Punctuated or granular and bright nuclei were considered apoptotic. Apoptotic nuclei were observed in cells treated with various concentrations of DDTC-copper complex, especially in cells treated with 5 or 10 μM DDTC-copper complex (Fig. 3C and D). Nuclear morphological change was not observed in control cells, or cells treated with copper or DDTC alone (Fig. 3C and D). Collectively, these results further support the conclusion that DDTC-copper complex can induce apoptosis in prostate cancer cells.

The effects of DDTC-copper complex on breast cancer cells. In order to determine the effects of DDTC-copper complex in other cancer cell lines, breast cancer cells, MCF-7 and MDA-MB-231, were treated with 10 μM copper, 10 μM DDTC, 1-10 μM DDTC-copper complex, or DMSO for 24 h, followed by measurement of levels of the proteasome activity,

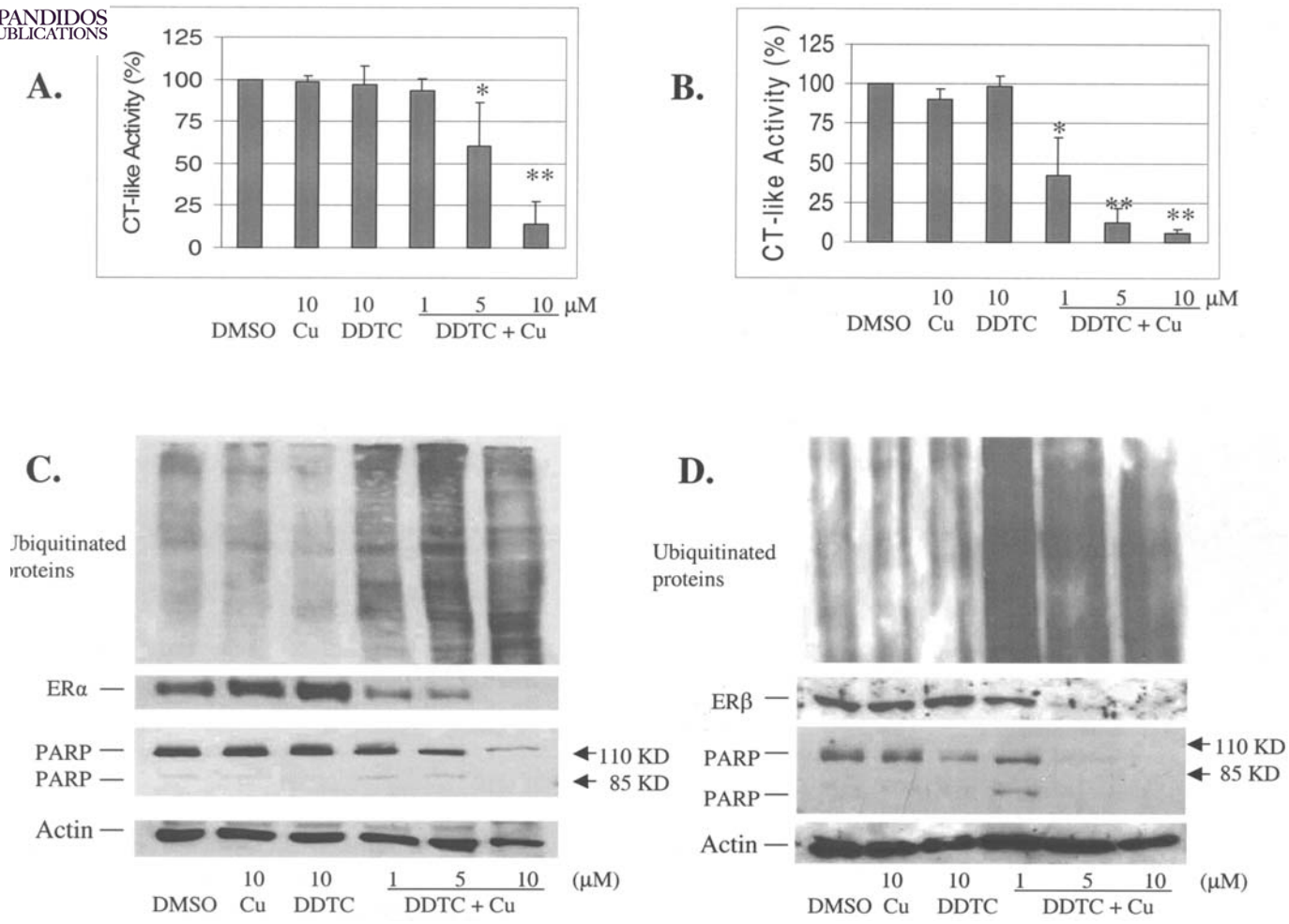

Figure 4. The effects of proteasome inhibition and apoptosis induction of DDTC-copper complex in breast cancer cells. (A) Chymotrypsin (CT)-like activity significantly decreased in MCF-7 cells treated with 5 or 10 μM of DDTC-copper complex compared with control cells ($^{*}P\leq0.05$, $^{**}P\leq0.01$). (B) Chymotrypsin-like activity significantly decreased in MDA-MB-231 cells treated with 5 or 10 μM of DDTC-copper complex ($^{**}P\leq0.01$). (C) Western blot analysis for accumulated ubiquitinated proteins, ERα expression, PARP cleavage, and actin expression in MCF-7 cells. (D) Western blot analysis for accumulated ubiquitinated proteins, ERß expression, PARP cleavage, and actin expression in MDA-MB-231 cells.

ER proteins and apoptosis. The DDTC-copper complex at the concentration of 5 and 10 μM significantly inhibited the proteasomal chymotrypsin-like activity by 40 and 85%, respectively, in MCF-7 cells (Fig. 4A), while at these two concentrations it inhibited the proteasomal chymotrypsin-like activity by 70 and 95%, respectively, in MDA-MB-231 cells (Fig. 4B), indicating that MDA-MB-231 cells are more sensitive to the DDTC-copper complex treatment. Consistently, higher levels of ubiquitinated proteins were accumulated in MDA-MB-231 cells than in MCF-7 cells after the treatment with the DDTC-copper complex at various concentrations (Fig. 4C and D). Neither copper alone nor DDTC alone resulted in these proteasomal-inhibitory effects (Fig. 4). These results demonstrated that DDTC-copper complex is also able to inhibit proteasome activity in breast cancer cells.

Regulation of estrogen receptors plays an important role in the growth of hormone-responsive and ER-positive breast cancer cells and tumors (26). In the same experiment, we investigated the effect of the DDTC-copper complex on expression of ER proteins. We observed that the expression of ERα protein in MCF-7 cells and of ERß protein in MDA-MB-231 cells was decreased in a DDTC-copper concentration-dependent fashion (Fig. 4C and D). In addition, the decreased expression of ERß in MCF-7 cells treated with various concentrations of DDTC-copper complex was observed (data not shown). However, ERα expression in MDA-MS-231 cells was not detectable.

In order to evaluate the ability of apoptosis induction of DDTC-copper in breast cancer cells, aliquots of the lysates of MCF-7 or MDA-MB-2321 cells in the same experiment were subjected to Western blot analysis with anti-PARP antibody. PARP cleavage was observed in both breast cancer cell lines treated with various concentrations of DDTC-copper complex (Fig. 4C and D), as we noted in prostate cancer cells (Fig. 2C and D). Neither copper nor DDTC alone was able to induce PARP cleavage in these breast cancer cells (Fig. 4C and D).

Furthermore, cellular spherical and detached changes were observed in both breast cancer cell lines treated with various concentrations of DDTC-copper complex, especially in cells treated with 5 or 10 μM DDTC-copper complex (Fig. 5A and B). Cellular morphological change was not observed in cells treated with DMSO, copper or DDTC alone (Fig. 5C and D). Also, apoptotic nuclei were observed in both cell lines treated with the DDTC-copper complex, especially when 5 or 10 μM

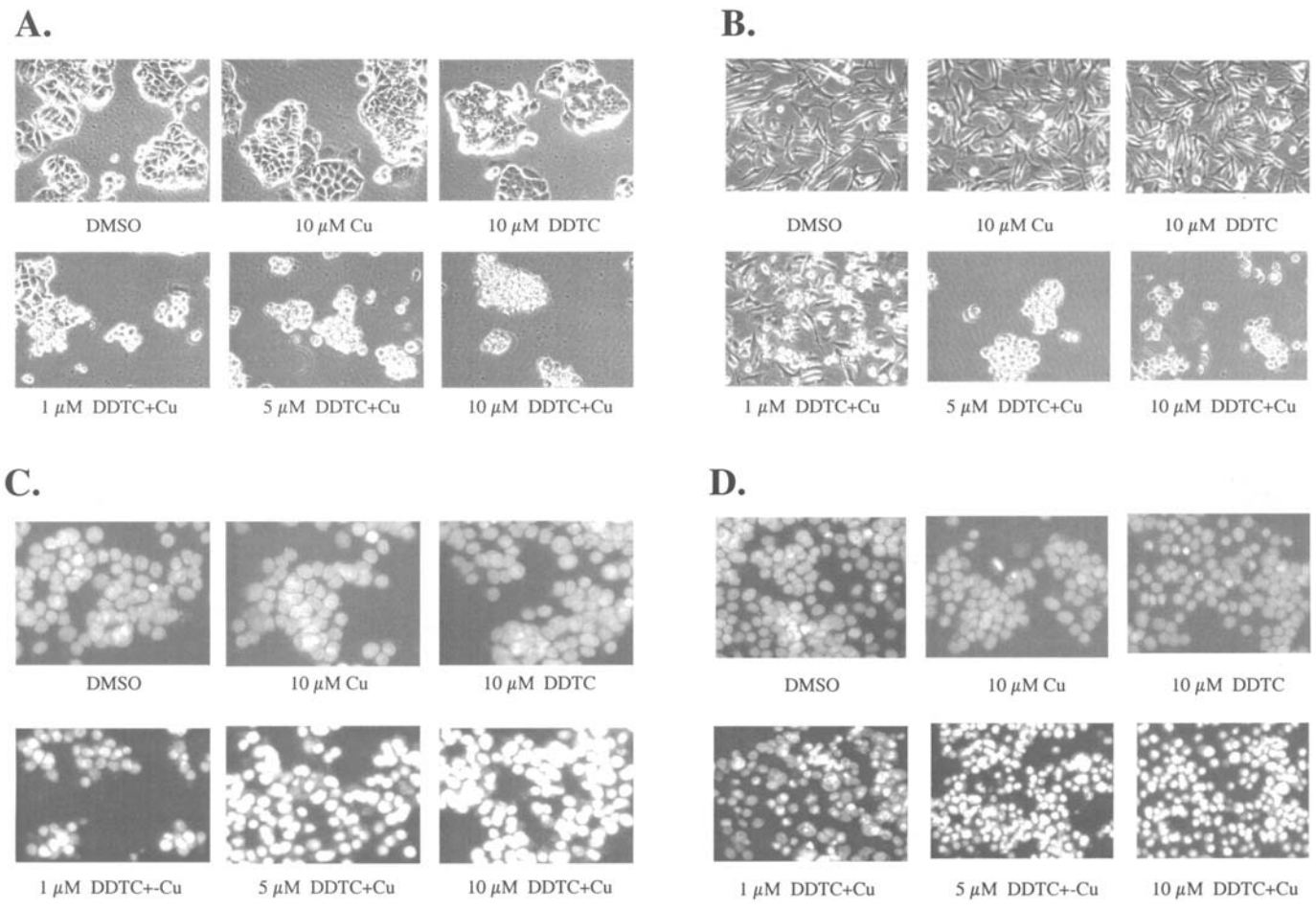

Figure 5. The cellular and nuclear morphological changes of breast cancer cells treated with DDTC-copper complex. (A) Cellular spherical and detached changes were shown in MCF-7 cells treated with various concentrations of DDTC-copper. (B) Cellular spherical and detached changes in MDA-MB-231 cells treated with DDTC-copper. (C) Punctuated, or granular, and bright nuclei indicating apoptotic nuclei are shown in MCF-7 cells treated with various concentrations of DDTC-copper complex. (D) Apoptotic nuclei in MDA-MB-231 cells treated with DDTC-copper complex.

complex was used (Fig. 5C and D). Nuclear morphological change was not observed in control cells or cells treated with copper or DDTC alone (Fig. 5). The results of cellular and nuclear morphology further support that the DDTC-copper complex can induce apoptosis in breast cancer cells.

Discussion

The ubiquitin-proteasome pathway has emerged as an attractive target for cancer therapy, since proteasome inhibition has produced encouraging anti-tumor activity in pre-clinical studies (27). Proteasomes perform a critical role in the degradation of key signaling molecules that promote cell cycle progression, cellular adhesion, and proliferation and induce anti-apoptotic pathways. Also, the proteasome is important in modulating the activity of the transcription factor nuclear factor-κB (NF-κB) (28,29). NF-κB is a transcription factor that plays an important role in cell proliferation and malignant transformation (30,31). Exposure of cancer cells to anticancer drugs, cytokines and radiation can induce the nuclear translocation and DNA-binding activity of NF-κB (32,33). Human cancer cells with induced NF-κB nuclear activity have demonstrated resistance to apoptosis induced by chemotherapy or radiotherapy (31,34,35). Through proteasome inhibition, the above mentioned critical tumor survival signaling cascades can be suppressed and several tumor suppressor proteins, such as p53, p21, p27, and Bax, can be stabilized, resulting in inhibition of cell cycle progression and induction of apoptosis (36). Bortezomib, the first proteasome inhibitor to enter clinical trials, inhibits tumor growth, induces apoptosis, and enhances the effect of chemotherapy in mice bearing human carcinoma cell xenografts (37,38). The better understanding of the role of the proteasome in the regulation of tumor cell growth has led to the development of new therapeutic approaches.

In this study, we found that DDTC spontaneously reacted with copper to form a DDTC-copper complex. The DDTC-copper complex at 5 μM inhibited proteasome activity and induced apoptosis in human prostate and breast cancer cells. In clinic DDTC is used as a therapeutic agent for treating metal poisoning and AIDS (15-18). Our new discovery that DDTC-copper can inhibit proteasome activity and induce apoptosis could be developed into a new therapeutic approach for cancer treatment. DDTC may bind a high level of copper in tumor tissues to form a DDTC-copper complex, leading to inhibition of the proteasome activity, induction of apoptosis,

fore suppression of tumor growth. Our data support pt of using accumulated copper in cancer cells and tissues as a target for chemotherapy, and provide a mechanistic interpretation for utilization of copper chelators in cancer treatment.

The proteasome pathway has been implicated as a major protease complex responsible for the turnover of most proteins in cells (39). In most cases, substrate recognition by the proteasome requires the attachment of multiple ubiquitin moieties to proteins targeted for degradation (40). Consistent with ubiquitin-dependent proteasome function, ubiquitination would serve as a targeting signal to direct AR or ER to the proteasome. In this study, we found that DDTC-copper complex could inhibit the expression of AR protein in prostate cancer cells and ER proteins in breast cancer cells. It is possible that the DDTC-copper complex inhibits transcription of AR or ER genes, resulting in down-regulation of AR or ER mRNAs and proteins and induction of apoptosis. The decrease in expression of AR or ER proteins may be regulated by inhibition of transcription. The other possible explanation of the decreased expression of AR or ER proteins is that DDTC-copper complex may affect other signal pathways, which cause cleavage of AR or ER proteins. Since caspases have emerged as the main players of the cell death program (41), the AR or ER cleavage may be regulated by the caspase pathway. It was reported that pharmacologic inhibition of caspase activation, an early event in apoptosis, protected cells from bortezomib-induced apoptosis (42). It should also be noted that many nuclear receptors are subjected to degradation via proteasome, and increased turnover of nuclear receptors and ubiquitin-proteasome system might be involved in the regulation of AR protein turnover under non-apoptotic conditions (37).

Our studies provide basic strategy for utilizing DDTC in prostate cancer treatment, since androgen/AR signal pathway plays a key role in the initiation and progression of prostate cancer. Any mechanism, which successfully blocks prostate cancer growth may provide a new target for designing novel therapeutic agent for prostate cancer treatment. Also, approximately half of all breast cancers are positive for ERα and are thought to be estrogen dependent (43). The regulation of transcriptional activation of ER is still unclear. The ubiquitin-proteasome proteolytic pathway is one mechanism involved in ER signaling (43). The proteasome inhibitor, Bortezomib, was reported to decrease the survival of both cultured MCF-7 cells and of EMT-6/Parent mouse mammary carcinoma xenograft tumors in a dose-dependent fashion (44). In the current study, the DDTC-copper complex as a proteasome inhibitor inhibited proteasomal chemotrypsin-like activity, decreased ER protein expression and induced apoptosis in breast cancer cells. This proteasome inhibitor should work by blocking degradation of the inhibitory protein IκB, thereby decreasing NFκB nuclear translocation. Most recently, we also reported that the clinically used anti-alcoholism drug and copper-binding agent disulfiram induces apoptotic cell death in breast cancer cultures and xenografts via inhibition of the proteasome activity (45). Collectively, our studies have demonstrated the potential use of these copper-binding compounds for treatment of human cancers.

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