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Pyrrolidine dithiocarbamate-zinc(II) and -copper(II) complexes induce apoptosis in tumor cells by inhibiting the proteasomal activity ☆

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ABSTRACT

Zinc and copper are trace elements essential for proper folding, stabilization and catalytic activity of many metalloenzymes in living organisms. However, disturbed zinc and copper homeostasis is reported in many types of cancer. We have previously demonstrated that copper complexes induced proteasome inhibition and apoptosis in cultured human cancer cells. In the current study we hypothesized that zinc complexes could also inhibit the proteasomal chymotrypsin-like activity responsible for subsequent apoptosis induction. We first showed that zinc(II) chloride was able to inhibit the chymotrypsin-like activity of a purified 20S proteasome with an IC_{50} value of 13.8 μM, which was less potent than copper(II) chloride (IC_{50} 5.3 μM). We then compared the potencies of a pyrrolidine dithiocarbamate (PyDT)-zinc(II) complex and a PyDT-copper(II) complex to inhibit cellular proteasomal activity, suppress proliferation and induce apoptosis in various human breast and prostate cancer cell lines. Consistently, zinc complex was less potent than copper complex in inhibiting the proteasome and inducing apoptosis. Additionally, zinc and copper complexes appear to use somewhat different mechanisms to kill tumor cells. Zinc complexes were able to activate calpain-, but not caspase-3-dependent pathway, while copper complexes were able to induce activation of both proteases. Furthermore, the potencies of these PyDT-metal complexes depend on the nature of metals and also on the ratio of PyDT to the metal ion within the complex, which probably affects their stability and availability for interacting with and inhibiting the proteasome in tumor cells.

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Introduction

Almost one century ago, zinc and copper were recognized as trace elements with important roles in various metabolic processes in living organisms (Labbe and Thiele, 1999; Andreini et al., 2006). Zinc is the second most abundant transitional metal ion in human body, essential for the proper function of different enzymes and for a tight control of gene expression (Maverakis et al., 2007). Moreover, the catalytic domain of all known matrix metalloproteinases contains a zinc-binding motif and an additional structural zinc ion required for the stability and the expression of the enzymatic activities (Marchenko et al., 2002; Malemud 2006).

Copper plays a central role in conserved processes, such as respiration, and in highly specialized processes, such as protein modifications. Additionally, angiogenesis, a process critical for tumor growth, requires copper as an essential cofactor that stimulates cytokine production, extracellular matrix degradation, and endothelial cell migration mediated by integrins (Eatock et al., 2000; Fox et al., 2001; Brewer, 2001). Having these important physiologic functions, it is not surprising that the concentrations of both zinc and copper are tightly regulated in an organism (Maverakis et al., 2007; Labbe and Thiele, 1999). Interestingly, disturbed zinc homeostasis and the elevated copper level have been reported in many types of cancer, including breast, prostate, lung, and brain (Geraki et al., 2002; Nayak et al., 2003; Diez et al., 1989; Yoshida et al., 1993).

Dithiocarbamates are a class of metal-chelating, antioxidant compounds with various applications in medicine for the treatment of bacterial and fungal infections, and possible treatment of AIDS (Schreck et al., 1992; Malaguarnera et al., 2003). One of the dithiocarbamates, pyrrolidine dithiocarbamate (PyDT), is a synthetic analog that has been reported to inhibit nuclear factor *kappa* B (NFκB) activation (Parodi et al., 2005; Schreck et al., 1992). However, when combined with either zinc(II) or copper(II) chloride, PyDT was shown to inhibit the ubiquitin–proteasome pathway (Kim et al., 2004; Daniel

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et al., 2005; Chen et al., 2005a). We have previously shown that PyDT-copper inhibits proliferation and induces apoptosis in cultured breast and prostate cancer cells by inhibiting proteasomal chymotrypsin-like activity (Daniel et al., 2005; Chen et al., 2005a). Based on that and similar location of zinc and copper in the periodic table, we hypothesized that the PyDT-zinc complex could have similar effect on the proteasome.

The ubiquitin–proteasome pathway is essential for many fundamental cellular processes, including the cell cycle, apoptosis, angiogenesis and differentiation (Orlowski and Dees, 2003; Landis-Piwowar et al., 2006). The proteasome contributes to the pathological state of several human diseases including cancer, in which some regulatory proteins are either stabilized due to decreased degradation or lost due to accelerated degradation (Ciechanover, 1998). 20S proteasome, the proteolytic core of 26S proteasome complex, contains multiple peptidase activities (including the chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing-like/PGPH) (Seemuller et al., 1995). It has been shown that inhibition of chymotrypsin-like but not trypsin-like proteasomal activity is a strong stimulus that induces apoptosis (An et al., 1998; Lopes et al., 1997). The possibility of therapeutically targeting the ubiquitin–proteasome pathway was met with great skepticism at the very beginning, since this pathway plays an important role in normal cellular homeostasis as well. However, after the demonstration that actively proliferating cancer cells are more sensitive to apoptosis-inducing stimuli, including proteasome inhibition, proteasome inhibitors became even more attractive (Dou and Li, 1999; Almond and Cohen, 2002; Orlowski and Dees, 2003; Adams, 2003).

To identify the component of the ubiquitin–proteasome pathway affected by PyDT-zinc, we first tested the ability of zinc(II) chloride to inhibit the chymotrypsin-like activity of the purified 20S proteasome, using copper(II) chloride as a comparison. We then compared the abilities of PyDT mixtures with zinc or copper to inhibit the cellular proteasome and induce apoptosis in various breast and prostate cancer cell lines. We found that PyDT-zinc(II) and PyDT-copper(II) mixtures and synthetic complexes exert their toxic effects against the cancer cells, associated with inhibition of the proteasomal chymotrypsin-like activity. We also found that both complexes induced apoptosis with different potencies, kinetics and molecular mechanisms. The potencies of PyDT-metal complexes depend not only on the nature of metals but also on the ratio of PyDT to the metal ion within the complex, which probably determines their stability and availability to interact with the tumor cellular proteasome.

Materials and methods

Materials. Pyrrolidine dithiocarbamate (PyDT), $CuCl_2$, $ZnCl_2$, 3-[4,5-dimethyltiazol-2-yl]-2.5-diphenyl-tetrazolium bromide (MTT), epidermal growth factor, insulin, dimethylsulfoxide (DMSO), 1,4-dithio-DL-threitol (DTT), *N*-acetyl-L-cysteine (NAC), cremophor and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640, DMEM/F12 (1:1), fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA) and MEME from ATCC (Manassas, VA). Purified rabbit 20S proteasome, and fluorogenic peptide substrates Suc-LLVY-AMC and Ac-DEVD-AMC (for the proteasomal chymotrypsin-like and caspase-3 activity, respectively) were from Calbiochem (San Diego, CA, USA). Mouse monoclonal antibody against human poly (ADP-ribose) polymerase (PARP) was purchased from BIOMOL International LP (Plymouth Meeting, PA). Mouse monoclonal antibodies against Bax (B-9), p27 (F-8), ubiquitin (P4D1), goat polyclonal antibody against actin (C-11), rabbit polyclonal antibody against IκB-α (C-15), and secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody against small subunit of μ- or m-calpains (calpain 1 and 2, respectively) (30 kDa) was purchased from Chemicon (Billerica, MA). Calpastatin was from Calbiochem and TUNEL assay kit from BD Biosciences Pharmingen, APO-DIRECT™ (San Diego, CA).

Synthesis of bis(1-pyrrolidinecarbodithioato-kS,kS')copper(II) [(PyDT)$_2$Cu] and bis(1-pyrrolidinecarbodithioato-kS,kS')zinc(II) [(PyDT)$_2$Zn]. (PyDT)$_2$Cu and (PyDT)$_2$Zn were synthesized following the literature (Shinobu et al., 1984; Orvig and Abrams, 1999). The powder of $CuCl_2$ or $ZnCl_2$ was added to a water solution of PyDT ammonium salt under vigorous stirring, in a metal-to-ligand molar ratio 1:2. This led to the immediate formation of a solid precipitant that was filtered, washed with water, and dried in a dessicator with P_4O_{10}, giving a final yield of 90–95%. Chemical composition of both final products was confirmed by the elemental analysis.

Cell culture and cell extract preparation. Human breast cancer MDA-MB-231 cells and prostate cancer PC-3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. Human breast cancer MCF10dcis.com (DCIS) and MCF-7 cell lines were grown as previously described (Daniel et al., 2004; Milacic et al., 2006). A whole cell extract was prepared as previously described (An et al., 1998).

In vitro proteasomal activity assay. Purified rabbit 20S proteasome (35 ng) was incubated with 20 μM of the fluorogenic peptide substrate Suc-LLVY-AMC in 100 μL assay buffer [20 mmol/L Tris–HCl (pH 7.5)] in the presence of $ZnCl_2$, $CuCl_2$, PyDT-$ZnCl_2$ mixture, PyDT-$CuCl_2$ mixture, (PyDT)$_2$Zn, (PyDT)$_2$Cu, or PyDT at various concentrations or equivalent volume of solvent DMSO as control. After 2 h incubation at 37 °C, inhibition of the proteasomal chymotrypsin-like activity was measured (Chen et al., 2005b).

Proteasome activity assay using breast and prostate cancer cells. MDA-MB-231, DCIS and MCF-7 breast cancer cells and PC-3 prostate cancer cells were grown to 70–80% confluency, treated under various conditions, harvested, and used for whole cell extract preparation. Ten (10) micrograms of cell extract was then used to determine the proteasomal chymotrypsin-like activity, as described above.

Cell proliferation assay. The MTT assay was used to determine the effects of various compounds on breast or prostate cancer cell proliferation. Cells were plated in a 96-well plate and grown to 70–80% confluency, followed by addition of each compound at the indicated concentrations. After 24 h incubation at 37 °C, inhibition of cell proliferation was measured as previously described (Milacic et al., 2006).

Cellular morphology analysis. A Zeiss (Thornwood, NY) Axiovert 25 microscope with phase contrast was used for cellular morphology imaging, as previously described (Daniel et al., 2005).

Caspase-3 activity assay. MDA-MB-231 breast cancer cells were treated with different concentrations of (PyDT)$_2$Zn or (PyDT)$_2$Cu for different time points, as indicated in the legends. The prepared whole cell extracts (30 μg per sample) were then incubated with 40 μM of Ac-DEVD-AMC in 100 μL assay buffer at 37 °C for at least 2 h. The release of the AMC groups was measured as previously described (Chen et al., 2005b).

Western blot analysis. Various breast and prostate cancer cell lines were treated as indicated in the figure legend. Cell lysates (50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ), as previously described (Chen et al., 2005b).

TUNEL assay and cell cycle analysis. Terminal deoxynecleotydyl transferase-mediated dUPT-biotin nick end-labeling (TUNEL) assay and cell cycle analysis were performed using the BD Biosciences Pharmingen, APO-DIRECT™ kit (San Diego, CA). Cells were treated with 20 μM of (PyDT)$_2$Zn or (PyDT)$_2$Cu for 16 h, harvested, fixed and stained according to the protocol.

Results

In vitro inhibition of the chymotrypsin-like activity of purified 20S proteasome by zinc(II) chloride and a PyDT-zinc(II) mixture

We have previously shown that copper dithiocarbamates could inhibit proteasomal activity in highly metastatic MDA-MB-231 breast cancer cells (Daniel et al., 2005). Zinc was also found to be involved in inhibition of the ubiquitin–proteasome pathway (Kim et al., 2004), but the detailed mechanism has not been completely understood.

We hypothesized that zinc is an inhibitor of the protesomal chymotrypsin-like activity. To test this hypothesis, we first incubated a purified rabbit 20S proteasome with zinc(II) chloride or copper(II) chloride (as a control) at various concentrations, followed by measurement of the proteasomal chymotrypsin-like activity. We found that zinc(II) chloride was able to inhibit the chymotrypsin-like activity of the purified 20S proteasome with an IC_{50} value of 13.8 μmol/L. Similar to previously reported (Daniel et al., 2004), the IC_{50} value of copper(II) chloride against the purified proteasomal chymotrypsin-like activity was determined as 5.3 μmol/L. Therefore, zinc(II) chloride also inhibits proteasomal chymotrypsin-like activity but with lower potency than copper(II) chloride.

To assess the proteasome-inhibitory effect of PyDT-zinc mixture, we mixed equal volumes of zinc(II) chloride or copper(II) chloride (as a comparison) with PyDT (Fig. 1A) and tested their proteasome-

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