Induction of tumor cell apoptosis by taurine Schiff base copper complex is associated with the inhibition of proteasomal activity

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Abstract. Schiff bases have been intensively investigated due to their antibacterial and antitumor properties. Copper is a cofactor essential for the tumor angiogenesis processes, whereas other transition metals are not. Consistently, high serum or tissue levels of copper were found in many types of human cancer including breast, prostate, colon, lung, and brain, supporting the idea that copper could be used as a novel selective target for cancer therapies. In the current study we hypothesize that a synthetic taurine Schiff base copper complex (Compound 1) could suppress tumor cell growth via the direct inhibition of proteasomal activity. Compound 1 potently inhibits the activity of purified 20S and 26S proteasome in human breast cancer MDA-MB-231 and leukemia Jurkat T cells. Inhibition of tumor cellular proteasomal activity by Compound 1 results in the accumulation of ubiquitinated protein and the proteasome target proteins p27 and Bax, followed by the induction of apoptosis. Our results strongly suggest that taurine Schiff base copper complexes, as potent proteasome inhibitors, have great potential to be developed into novel anticancer drugs.

Introduction

Since the discovery of cisplatin for cancer treatment, numerous transition metal complexes have been synthesized and screened for their anticancer properties (1,2). Copper, is a cofactor essential for the tumor angiogenesis processes, whereas other transition metals are not (3-5). Consistently, high levels of copper have been found in many types of human cancer (6,7). A specific amount of local copper appears to be required for angiogenesis to occur, although the involved mechanism remains unknown. Therefore, a novel concept was developed suggesting that the drugs targeting tumor cellular copper may be more effective and less toxic. This prompted researchers to develop copper-based compounds with potential use as anticancer drugs.

The ubiquitin-proteasome pathway, which plays an important role in regulating cell proliferation and cell death, (8,9) has been extensively studied in human cancers. The proteasome is a massive multicatalytic protease responsible for degrading a large number of cellular proteins. These target proteins are first tagged with ubiquitin in order to be degraded by the proteasome (8,9). Several regulatory proteins involved in cell cycle and apoptosis processes, such as cyclins, bcl-2 family members, and p53, are degraded by the ubiquitin-proteasome pathway (10,11). The eukaryotic proteasome 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) (12). We and others reported that the inhibition of the proteasomal chymotrypsin-like, but not trypsin-like activity, is associated with the induction of tumor cell apoptosis (13,14). Recently, our laboratory reported that some copper complexes can potently and selectively inhibit the chymotrypsin-like activity of the proteasome, followed by the induction of apoptosis *in vitro* and *in vivo* (15-19).

Schiff base (or azomethine), named by the chemist Hugo Schiff, is a functional group that contains a double bond between carbon and nitrogen (Fig. 1). Schiff base ligands are able to coordinate metals through imine nitrogen and another group and to stabilize many different metals in various states of oxidation (20). Schiff bases and their metal complexes have been intensively investigated due to their antibacterial and antitumor properties (21-23).

Taurine (Fig. 1) is an example of an amino acid which has become increasingly popular as an ingredient in dietary

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supplements and functional foods and beverages. It has been reported that Schiff bases derived from taurine have manifold coordination modes (24). Such an aromatic-ring stacking interaction is an important characteristic of ternary complex, which can stabilize the double-helical structure (25) and the interaction between anticancer drugs and DNA.

In the current study, a taurine Schiff base copper complex, (Compound 1) (Fig. 1), has been synthesized and evaluated for its proteasome-inhibitory and apoptosis-inducing activities in human breast cancer and leukemia cells. We reported that this copper complex can potently inhibit the cellular proteasomal activity and induce apoptosis in dose- and time-dependent manners. Our study reinforces the idea that proteasome-targeted copper compounds have great potential to be developed into novel anticancer drugs (16,18,19).

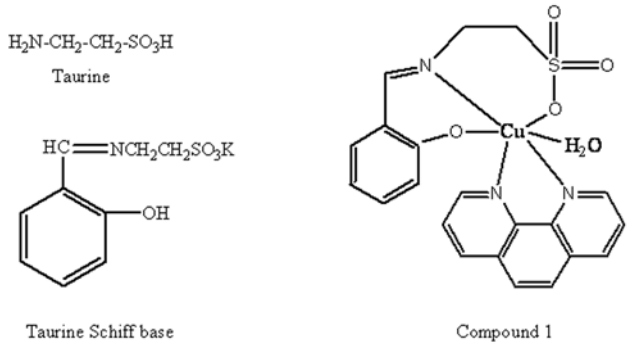

Figure 1. Chemical structures of taurine, taurine Schiff base and Compound 1.

Materials and methods

Materials. Compound 1 was synthesized as described previously (26). Fetal bovine serum was purchased from Tissue Culture Biologicals (Tulare, CA). DMEM/F-12 and RPMI-1640 medium, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). Purified rabbit 20S proteasome, fluorogenic peptide substrate Suc-LLVY-AMC and Ac-DEVD-AMC were obtained from Calbiochem, Inc. (San Diego, CA). 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), DMSO and other chemicals were purchased from Sigma-Aldrich (St. Louis). Mouse monoclonal antibody against human poly(ADP-ribose) polymerase (PARP) was from Biomol International LP (Plymouth Meeting, PA). Mouse monoclonal antibodies against Bax (B-9), p27 (F-8) and ubiquitin (P4D1), and goat polyclonal antibody against actin (C-11) and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell cultures and whole-cell extract preparation. MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/F-12 media. Jurkat T cells were cultured in RPMI-1640 media. The media were supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. All cells were maintained at 37˚C and 5% CO_2. A whole-cell extract was prepared as previously described (13,16).

Inhibition of purified 20S proteasome activity by Compound 1. The chymotrypsin-like activity of purified 20S proteasome was measured as previously described (16). Briefly, 17.5 ng purified 20S proteasome were incubated in 100 μl assay buffer (50 mM Tris-HCl, pH 7.5) with or without different concentrations of Compound 1 and 20 μM fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) for 2 h at 37˚C. After incubation, production of hydrolyzed AMC groups was measured with a Wallac Victor 3 multilabel counter with an excitation filter of 365 nm and an emission filter of 460 nm.

Proteasomal chymotrypsin-like activity in cell extracts. Whole-cell extracts (4 μg) were incubated for 2 h at 37˚C in 100 μl assay buffer (50 mM Tris-HCl, pH 7.5) with 20 μM fluorogenic substrate Suc-LLVY-AMC. This was followed by the measurement of AMC group release, as previously described.

MTT and trypan blue exclusion assays. MDA-MB-231 cells, seeded in triplicate in a 96-well plate and grown until 70-80% confluence, were treated with indicated agents for 16 h. This was followed by MTT assay as previously described (17). Jurkat T cells were treated with the solvent (DMSO) and the indicated compound for 24 h. Then we mixed 20 μl of cell suspension with 20 μl of 0.4% trypan blue dye before injecting it into a hemocytometer and counting. The number of cells that absorbed the dye and turned a purple color were counted as dead cells, from which the percentage of non-viable cell number to total cell number was calculated.

Cellular morphologic analysis. A Zeiss Axiovert-25 microscope was used for all microscopic imaging with phase contrast for cellular morphology, and cells that become round and detached were considered as apoptotic cells.

Western blot analysis. Equal amounts of cell lysate (40 μg) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and blotted with indicated antibodies followed by visualization with the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Results

Compound 1 inhibits the chymotrypsin-like activity of purified 20S proteasome. Previously we reported that certain copper complexes have potent proteasome-inhibitory properties in cancer cells (15-19). To examine whether the novel taurine Schiff base copper complex, Compound 1 (Fig. 1), is capable of inhibiting the proteasome activity, we incubated Compound 1 at various concentrations with a purified rabbit 20S proteasome. The results showed that Compound 1 inhibited the chymotrypsin-like activity of the purified 20S proteasome with an IC_{50} value of 12±1.5 μM.

Concentration-dependent proteasome inhibition and apoptosis induction by Compound 1. To determine whether Compound 1 inhibits tumor cellular proteasomal activity,

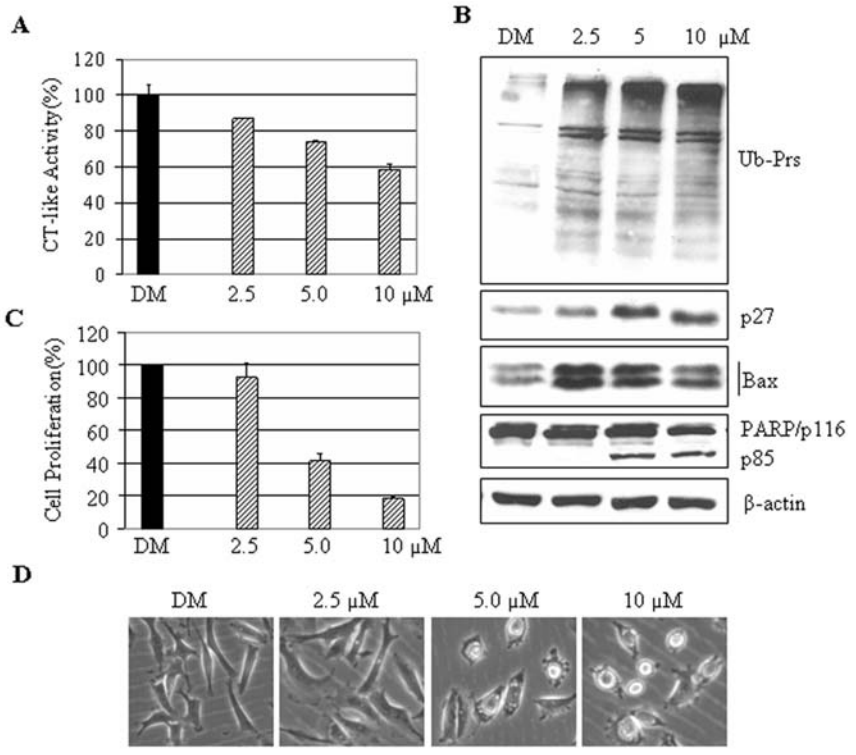

Figure 2. Dose response experiment in breast cancer MDA-MB-231 cells. MDA-MB-231 cells were treated with either solvent DMSO (DM) or indicated concentrations of Compound 1 for 4 h [A, B (Ubiquitinated proteins, p27 and Bax)] or 16 h [B (PARP), C]. This was followed by measuring inhibition of the proteasomal CT-like activity using the fluorescent substrate Suc-LLVY-AMC (A), Western blot analysis using specific antibodies to ubiquitin, p27, Bax, PARP, ß-actin (as loading control) (B), MTT assay (C) and morphological changes (D).

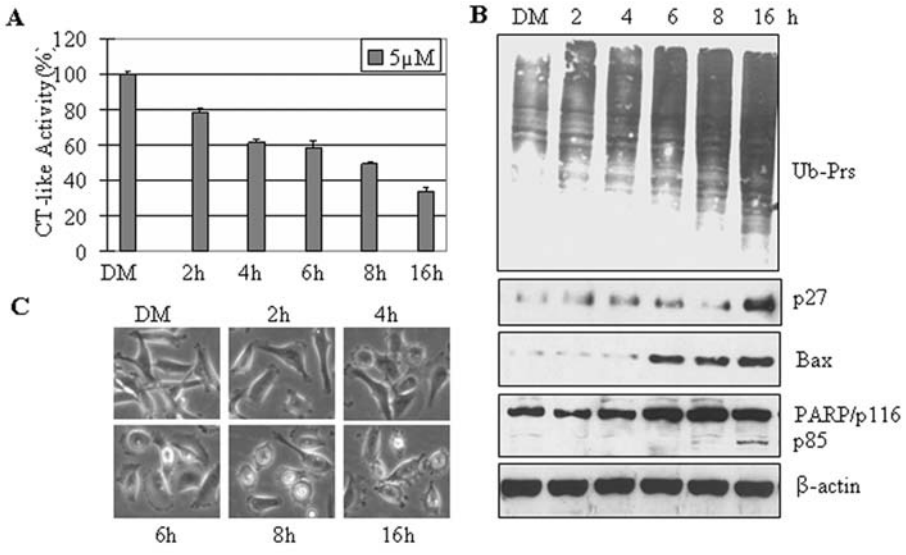

Figure 3. Kinetic effects of Compound 1 on MDA-MB-231 cells. MDA-MB-231 cells were treated with 5 μM of Compound 1 for the indicated hours. This was followed by chymotrypsin-like activity assay using cell extracts (A), Western blot analysis with antibodies to ubiquitin, p27, Bax, PARP, and ß-actin (B) and morphological changes (C).

human breast cancer MDA-MB-231 cells were treated with Compound 1 for 4 or 16 h. Proteasome inhibition and apoptosis induction were measured. We found that treatment with this copper complex at 2.5, 5 and 10 μM for 4 h resulted in 12, 25 and 41% inhibition of proteasomal activity, respectively (Fig. 2A). Consistently, accumulation of ubiquitinated proteins occured and the proteasome target proteins p27 and Bax were observed after 4 h treatment in the treated cells (Fig. 2B). These results confirm that Compound 1 inhibits cellular proteasomal activity in breast cancer MDA-MB-231 cells.

It has been demonstrated that the inhibition of the proteasomal chymotrypsin-like activity is associated with the induction of tumor cells growth arrest and/or apoptosis (15-

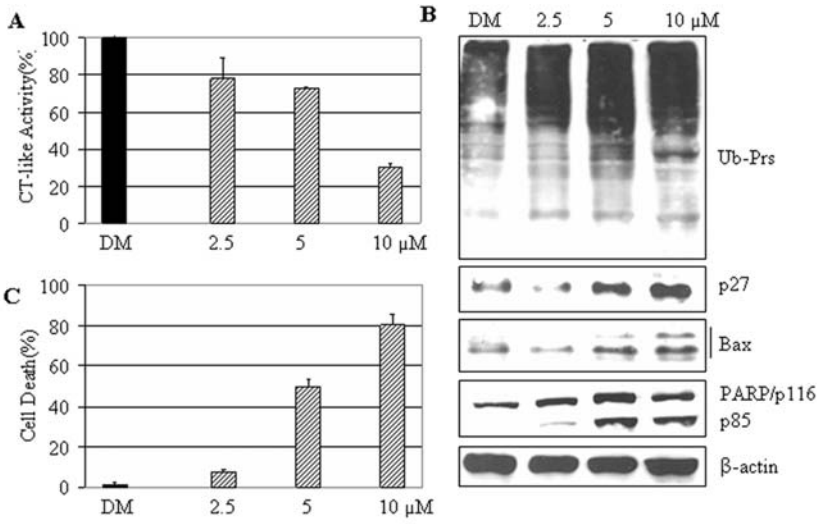

Figure 4. Dose response experiment in leukemia Jurkat T cells. Jurkat T cells were treated with the solvent (DMSO) and the indicated concentrations of Compound 1 for 24 h. This was followed by measuring the inhibition of the proteasomal CT-like activity (A), Western blot analysis using specific antibodies to ubiquitin, p27, Bax, PARP, ß-actin (B), and trypan blue dye exclusion assay (C).

19). To determine whether proteasome inhibition by Compound 1 causes suppression of cell proliferation, MDA-MB-231 cells were treated for 16 h with different concentrations of Compound 1 or an equal volume of the vehicle DMSO, followed by performance of MTT assay. We found that Compound 1 at 2.5, 5 and 10 μM inhibited the proliferation of MDA-MB-231 cells in a concentration-dependent manner, by 8, 58, and 82%, respectively (Fig. 2C).

To determine whether inhibition of growth is due to the induction of apoptotic cell death, two indices for apoptosis, PARP cleavage and morphological changes, were measured in the same experiment. After 16 h treatment, PARP cleavage was detected in the cells treated with Compound 1 at 5 and 10 μM (Fig. 2B). At 16 h, apoptosis-associated morphological changes (i.e., shrunken and detached) were observed in the cells treated with 5 μM Compound 1 (Fig. 2D), which was further increased to ~100% when 10 μM Compound 1 was used (Fig. 2D). These results demonstrate that Compound 1 is able to induce apoptosis in breast cancer cells.

Compound 1 induces a time-dependent proteasome inhibition and apoptosis in MDA-MB-231 cells. If proteasome inhibition was responsible for apoptosis induction by Compound 1, we would expect the proteasomal activity to be inhibited before the apoptotic events occurred. In a kinetic experiment, we found that the proteasome inhibition by Compound 1 started as early as 2 h, as evident by a 23% decrease in the proteasome activity level (Fig. 3A) and an increase in the level of ubiquitinated proteins (Fig. 3B). From 4 to 16 h, the proteasomal chymotrypsin-like activity level was further decreased (Fig. 3A) while the ubiquitinated proteins further accumulated (Fig. 3B). Consistent with proteasome inhibition, the levels of proteasome target proteins p27 and Bax were increased in a time-dependent manner (starting at 4 and 6 h, respectively; Fig. 3B). Importantly, cell death was not observed until 16 h of treatment with Compound 1, as shown by lack of apoptosis-specific PARP cleavage (Fig. 3B) and lack of cellular morphological changes before 16 h (Fig. 3C). Apoptosis

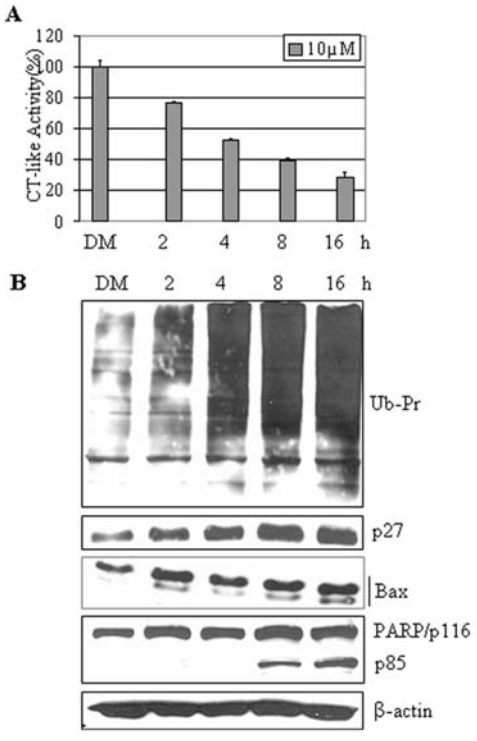

Figure 5. Kinetic effects of Compound 1 on Jurkat T cells. Jurkat T cells were treated with 10 μM of Compound 1 for the indicated hours. This was followed by chymotrypsin-like activity assay using cell extracts (A) and Western blot analysis for ubiqutinated proteins, p27, Bax, PARP and ß-actin (B).

started after the cells were treated for 16 h, as demonstrated by the production of PARP p85 cleavage fragment (Fig. 3B) and cellular round up and detachment (Fig. 3C). These results suggest that apoptosis induced by Compound 1 is a consequential event of proteasome inhibition.

Inhibition of proteasomal activity by Compound 1 in human leukemia Jurkat T cells is associated with the induction of apoptosis. In order to test whether Compound 1 can inhibit

cellular proteasomal activity and induce apoptosis in different cancer cells, we used human leukemia Jurkat T cells for dose- and time-dependent experiments. In the first experiment, Jurkat T cells were treated with various concentrations of Compound 1 for 24 h. This was followed by proteasome inhibition and apoptosis assays. We found that Compound 1, when used in Jurkat T cells, caused a dose-dependent inhibition of the proteasomal chymotrypsin-like activity (Fig. 4A). Consistently, we observed a dose-dependent increase in levels of ubiqutinated proteins and proteasome target proteins p27 and Bax (Fig. 4B). This was accompanied by dose-dependent apoptotic cell death: Compound 1 at 2.5, 5 and 10 μM induced ~10, 50 and 81% cell death, respectively (Fig. 4C), and at 5 and 10 μM induced significant production of PARP p85 cleavage fragment (Fig. 4B).

In the kinetic experiment using Jurkat T cells, the proteasomal chymotrypsin-like activity was inhibited by 24% after 2 h treatment with 10 μM Compound 1 (Fig. 5A), associated with increased levels of ubiqutinated proteins, p27 and Bax at this time (Fig. 5B). From 4 to 16 h, the proteasomal activity was further decreased and ubiquitinated proteins and the proteasome target proteins were further accumulated (Fig. 5A and B). However, the cleavage of the PARP protein was not detected before 8 h (Fig. 5B), at least 6 h later than proteasome inhibition (Fig. 5B vs. 5A). The results clearly demonstrate that Compound 1 inhibits cellular proteasome and causes apoptosis in human leukemia cells.

Discussion

Proteasome inhibition and anti-angiogenesis have been found to be novel approaches to cancer therapy due to the fact that the cancer cells are much more dependent on these activities and processes than normal cells (9,27-31). Copper, is an essential cofactor and requisite for the tumor angiogenesis processes (3-5), whereas other trace metals in the body, are not. It has been found that the cellular copper is not all protein-bound and can be stored in membranes (32). Therefore copper-based drugs have potential use in cancer therapies.

In the current study, we determined the proteasome-inhibitory and apoptosis-inducing abilities of a novel synthetic Schiff base copper complex, Compound 1 (Fig. 1). We hypothesized that this novel copper complex may inhibit the proteasomal activity, leading to apoptosis induction. In order to test this hypothesis, we performed a cell-free proteasomal activity assay using a purified 20S proteasome and found that Compound 1 potently inhibited the chymotrypsin-like activity of the purified 20S proteasome with an IC_{50} of 12 μM.

After we showed that Compound 1 inhibited the purified proteasomal chymotrypsin-like activity, we tested its effect in intact breast cancer MDA-MB-231 cells and found similar inhibitory effects. Proteasomal inhibition in MDA-MB-231 cells by this compound was supported by decreased proteasomal activity (Figs. 2A and 3A) and increased levels of ubiquitinated proteins and the proteasome target proteins p27 and Bax (Figs. 2B and 3B) in dose- and time-dependent experiments. These findings indicate that Compound 1 directly targets the cellular proteasome in breast cancer cells.

We and others have reported that various proteasome inhibitors potently induce apoptosis (13,14,29,30). Therefore, we investigated whether Compound 1 behaved similarly. First we measured its antiproliferation activity by the MTT assay, and found that Compound 1 suppresses the proliferation of breast cancer MDA-MB-231 cells in a dose-dependent fashion (Fig. 2C). From the kinetic experiment, we noted that the accumulation of ubiquitinated proteins and proteasome target proteins p27 and Bax were increased prior to apoptosis induction. The apoptosis-specific PARP cleavage and morphological changes were also found in dose- and time-dependent manners (Fig. 3B and C). These findings further support the conclusion that inhibition of the chymotrypsin-like activity of the proteasome by a specific inhibitor is sufficient to induce apoptosis (13,16).

To determine whether Compound 1 can inhibit proteasome and induce apoptosis in other cancer cells, we treated human leukemia Jurkat T cells with Compound 1. Results of a trypan blue assay showed that this compound induced Jurkat T cell death in a dose-dependent manner (Fig. 4C). Consistently, Compound 1 potently inhibits the chymotrypsin-like activity (Figs. 4A and 5A) and significantly accumulates levels of ubiquitinated proteins and proteasome target proteins p27 and Bax (Figs. 4B and 5B). Induction of apoptosis (Figs. 4B and 5B), in dose- and time-dependent manners, followed. Therefore, Compound 1 is a potent proteasome inhibitor and apoptosis inducer in leukemia Jurkat T cancer cells as well as in breast cancer MDA-MB-231 cells.

In summary, we have identified that the tumor proteasome is a target of the novel copper complex Compound 1. We have shown that the inhibition of the proteasomal activity (especially, chymotrypsin-like activity) by this compound, can strongly induce apoptosis in the cultured breast cancer and leukemia cells. There are two possible mechanisms responsible for this compound targeting and inhibiting the proteasome. First, this compound may bind to the proteasome and inhibit its activity. Another possibility is that this compound might transfer more copper into the cells and that copper may oxidise the proteasome, causing inactivation. However, more detailed studies are required to confirm these potential mechanisms. Our study reinforces the idea that proteasome-targeted copper compounds have great potential to be developed into novel anticancer drugs.

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