Disulfiram, a Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts via Inhibition of the Proteasome Activity

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Abstract

Disulfiram (DSF), a member of the dithiocarbamate family capable of binding copper and an inhibitor of aldehyde dehydrogenase, is currently being used clinically for the treatment of alcoholism. Recent studies have suggested that DSF may have antitumor and chemosensitizing activities, although the detailed molecular mechanisms remain unclear. Copper has been shown to be essential for tumor angiogenesis processes. Consistently, high serum and tissue levels of copper have been found in many types of human cancers, including breast, prostate, and brain, supporting the idea that copper could be used as a potential tumor-specific target. Here we report that the DSF-copper complex potently inhibits the proteasomal activity in cultured breast cancer MDA-MB-231 and MCF10DCIS.com cells, but not normal, immortalized MCF-10A cells, before induction of apoptotic cancer cell death. Furthermore, MDA-MB-231 cells that contain copper at concentrations similar to those found in patients, when treated with just DSF, undergo proteasome inhibition and apoptosis. In addition, when administered to mice bearing MDA-MB-231 tumor xenografts, DSF significantly inhibited the tumor growth (by 74%), associated with in vivo proteasome inhibition (as measured by decreased levels of tumor tissue proteasome activity and accumulation of ubiquitinated proteins and natural proteasome substrates p27 and Bax) and apoptosis induction (as shown by caspase activation and apoptotic nuclei formation). Our study shows that inhibition of the proteasomal activity can be achieved by targeting tumor cellular copper with the nontoxic compound DSF, resulting in selective apoptosis induction within tumor cells. (Cancer Res 2006; 66(21): 10425-33)

Introduction

Copper is an essential trace metal for animals. The amount of copper in an organism is very tightly regulated (1–3). Many critical enzymes and transcription factors require copper for their activities (1–3). Angiogenesis, the growth of a tumor blood supply, is essential for tumor growth, invasion, and metastasis (4, 5). Tumors without an additional blood supply do not grow larger than 1 to 2 mm³ (6). Molecular processes of angiogenesis include stimulation of endothelial growth by tumor cytokine production (i.e., vascular endothelial growth factor) and the requirement of copper (but not other trace metals) as an essential cofactor (3–6). Consistently, high serum or tissue levels of copper were found in many types of human cancers including breast, prostate, colon, lung, and brain (7–13), although the involved mechanism remains unknown. The above information suggests that copper could be used as a novel selective target for cancer therapies. Along this line, therapies with the strong copper chelator tetrathiomolybdate (TM) are well tolerated and copper elimination can stabilize advanced kidney cancer (14, 15), showing the clinical feasibility of this approach.

The proteasome-mediated degradation pathway has been considered as an important target for anticancer drug development. The proteasome inhibitor bortezomib (Velcade, PS-341) has been used in clinical trials and its antitumor activity has been reported in a variety of tumor models (16–18). The ubiquitin/proteasome-mediated proteolytic system controls the turnover of critical regulatory proteins involved in several cellular processes such as cell cycle and apoptosis (19–21). This degradation pathway includes two distinct steps: ubiquitination and degradation. Ubiquitination is the step after which the target protein can be selectively recognized from other proteins by the 26S-proteasome complex. Degradation of proteins occurs on the large 26S-proteasome complex in an ATP-dependent manner (19–21). The eukaryotic proteasome contains at least three known catalytic activities: chymotrypsin-like, trypsin-like, and caspase-like or peptidyl-glutamyl peptide-hydrolyzing–like activities (22). Our laboratory and others have reported that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of apoptosis in tumor cells (23–25).

Disulfiram (DSF; Fig. 1A) is a member of the dithiocarbamate family comprising a broad class of molecules possessing an R₁R₂NCS(S)SR₃ functional group, which gives them the ability to complex metals and react with sulphydryl groups (26–28). DSF, an irreversible inhibitor of aldehyde dehydrogenase, is one of the two drugs approved by the Food and Drug Administration (FDA) for treatment of alcoholism (28). Clinical trials have shown the efficacy of DSF with no toxicity (28). Several studies have shown that DSF and its metabolites can potentiate the effect of some anticancer drugs (29, 30). However, the precise mechanisms are still unknown.

We have reported that certain classes of copper-containing compounds act as potent proteasome inhibitors (3, 25). Because DSF can also bind copper (31, 32), we hypothesized that the DSF-copper complex is a proteasome inhibitor. We report here that, indeed, DSF is capable of binding copper and forming a new complex, which has proteasome inhibitory and apoptosis-inducing activities when tested in cultured human breast cancer, but not...
normal/immortalized, cells. In addition, after pretreatment with copper chloride to increase cellular copper concentration, breast cancer MDA-MB-231 cell were sensitive to DSF-induced proteasome inhibition and apoptosis induction. Furthermore, when administered to mice bearing breast cancer MDA-MB-231 xenografts, DSF potently inhibited tumor growth, associated with \textit{in vivo} proteasome inhibition and apoptotic cell death. Our study further reinforces the idea that inhibition of the proteasome activity can be achieved by targeting tumor cellular copper and suggests the potential use of DSF as a novel anticancer drug.

**Materials and Methods**

**Materials.** DSF, CuCl₂, ammonium tetra(thiomolybdate) (TM), Hoechst 33258, and cremophor were purchased from Sigma-Aldrich (St. Louis, MO). Purified rabbit 20S proteasome, fluorogenic peptide substrate Suc-LLVY-AMC, and Ac-DEVD-AMC were obtained from Calbiochem, Inc. (San Diego, CA). Peptide substrate Z-GGL-AMC was from Biomol International LP (Plymouth Meeting, PA). Apoptag Peroxidase In Situ Apoptosis Detection Kit was from Chemicon International, Inc. (Temecula, 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 supplemented with 10% fetal bovine serum. MCF10A (normal, derived from benign human breast tissue) and MCF10DCIS.com (human malignant breast cells) cells were obtained and cultured as previously described (33). All cells were maintained at 37°C and 5% CO₂. A whole-cell extract was prepared as previously described (23, 25).

**Inhibition of purified 20S proteasome activity by copper chloride and the DSF-copper mixture.** The chymotrypsin-like activity of purified rabbit 20S proteasome was measured as previously described (19). Briefly, 17.5 ng of purified 20S proteasome was incubated in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 7.5) with or without different concentrations of copper chloride, DSF, or the DSF-copper mixture and 10 μmol/L of fluorogenic peptide substrate Suc-LLVY-AMC for 24 hours, followed by Western blot analysis for accumulation of ubiquitinated proteins (Ub-Prs) and PARP cleavage. MTT assay. MDA-MB-231 cells were treated with 20 μmol/L of copper (Cu), DSF, TM, DSF-copper complex, or TM-copper complex (TM-Cu) for 24 hours, followed by Western blot analysis and MTT assay as described in Materials and Methods. Columns, mean of three experiments; bars, SD. D, DSF-copper induces apoptosis in breast cancer cells. As described in (B), treated MDA-MB-231 cells were used for determination of cellular and nuclear apoptotic changes. Left, cellular morphologic changes (spherical and detached cells). Right, apoptotic nuclear changes were shown by Hoechst 33258 staining (punctuated, granular, and brighter nuclei).
activity) for 2 hours at 37°C. After incubation, production of hydrolyzed AMC groups was measured with a Wallac Victor3 multilabel counter with an excitation filter of 365 nm and an emission filter of 460 nm.

**Proteasomal chymotrypsin-like and caspase-3 activity assays in cell extracts.** Whole-cell extracts (10 μg) of cells treated as indicated or tumor tissue extracts (10 μg) from human breast tumor xenografts were incubated for 1 hour at 37°C in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 7.5) with 20 μmol/L fluorogenic substrate Suc-LIY-AMC (for the proteasomal chymotrypsin-like activity in cell extracts) or Z-GGL-AMC (for specific chymotrypsin-like activity in tumor tissues) or Ac-DEVD-AMC (for caspase-3 in tumor tissues) as previously described (34).

**Cell proliferation assay.** MDA-MB-231 cells were seeded in triplicate in a 96-well plate and grown until 70% to 80% confluence, followed by treatment with indicated agents for 24 hours. After that, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done as previously described (34).

**Cellular and nuclear morphologic analysis.** A Zeiss Axiovert 25 microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology after Hoechst 33258 staining as previously described (34). Punctuated, granular, and brightly stained nuclei were considered apoptotic.

**Western blot analysis.** The cell or tissue extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was done using specific antibodies against ubiquitin, p27, Bax, or actin (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or poly(ADP-ribose) polymerase (PARP; Biomol International), followed by visualization with the enhanced chemiluminescence reagent (Amersham Biosciences, Piscatway, NJ).

**Human breast tumor xenograft experiments.** Five-week-old female athymic nude mice were purchased from Taconic Research Animal Services (Hudson, NY) and housed under pathogen-free conditions according to Wayne State University animal care guidelines. The protocols of animal experiments were reviewed and approved by Institutional Laboratory Animal Care and Use Committee of Wayne State University. MDA-MB-231 cells (5 × 10⁶) were injected s.c. at one flank of the mice. Tumor size was measured every other day. Tumor volume (V) was determined by the equation: \( V = (L \times W^2) \times 0.5 \), where L is the length and W is the width of the tumor. When xenografts reached volumes of ~200 mm³, the mice bearing tumors were randomly assigned to control or DSF groups (n = 10), and administered daily using either solvent control (PBS/cremophor/ DMSO/ethanol, 7.5:1:5:0:5:0.5) or 50 mg/kg/d DSF. When the control tumors reached ~1,600 mm³ (on day 29), the experiment was terminated and the mice were sacrificed. The tumors were removed and photographed and the tumor tissues were then used for multiple assays to measure proteasome inhibition and apoptotic cell death.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay.** Tumor tissues were paraffin embedded and stained according to the instruction of the manufacturer. Briefly, after deparaffinization and hydration, the tissue was incubated with Working Strength TdT Enzyme, Working Strength Stop/Wash Buffer, conjugated with antidigoxigenin, and then stained with peroxidase substrate. Finally, the tissue was mounted under a glass coverslip in Permount and viewed under a microscope.

**Immunohistochemistry.** Tumor tissues were paraffin embedded. After deparaffinization and hydration, the slide was blocked its endogenous peroxidase by 3% hydrogen peroxide, incubated with primary antibody p27 (1:200 Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), then with biotinylated secondary antibody, antirabbit immunoglobulin G (H + L), followed by incubation in ABC reagent (Avidin and Biotinylated horseradish peroxidase Complex, DAKO Labs, Cambridgehire, United Kingdom). Finally, the slide was mounted with 3,3′-diaminobenzidine and visualized under a microscope.

**H&E staining assay.** Paraffin-embedded sample slides were deparaffinized and hydrated, and then stained with hematoxylin for 1 minute. After rinsing, the slides were then stained with eosin for 1 minute, followed by more rinse, and coverslips were mounted onto slides with Permount.

**Statistical analysis.** Statistical analysis was done with Microsoft Excel software. Student’s t test for independent analysis was applied to evaluate differences between treatment and control.

**Results**

The DSF-copper complex inhibits the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in breast cancer cells. It has been shown that DSF is able to bind copper (31, 32). Indeed, when a solution of DSF was mixed with a solution of CuCl₂ at 1:1 ratio, dramatic color change was observed, from light blue to dark green (data not shown). This indicates that a chemical reaction has occurred that involves formation of a DSF-copper complex.

We have previously reported that certain copper complexes act as proteasome inhibitors in cancer cells (3, 25, 34). To examine whether the complex of DSF-copper is capable of inhibiting the proteasome activity, we incubated CuCl₂, DSF alone, or the DSF-copper mixture at various concentrations with a purified rabbit 20S proteasome. The results showed that both CuCl₂ and DSF-copper could inhibit the chymotrypsin-like activity of the purified 20S proteasome with an IC₅₀ value of ~7.5 μmol/L for both (Fig. 1A). In a sharp contrast, DSF alone at even 20 μmol/L had little effect (Fig. 1A). This is consistent with our hypothesis that DSF is able to carry the copper ion into tumor cells and prevent copper from interacting with many nonspecific proteins, and that it is the copper ion that is responsible for inhibiting the proteasome molecule (see below).

To further evaluate this hypothesis, human breast cancer MDA-MB-231 cells were treated with 20 μmol/L copper alone, DSF alone, the DSF-copper complex, TM, or the TM-copper complex (as a comparison). Cells treated with DMSO were used as control. After 24-hour treatment, the cells were collected and lysates were prepared for measurement of proteasome inhibition by decreased levels of the proteasomal chymotrypsin-like activity and accumulation of ubiquitinated proteins. The proteasomal chymotrypsin-like activity was decreased by >95% in the breast cancer cells treated with the DSF-copper complex, compared with the vehicle control-treated cells (Fig. 1B, top). In contrast, neither copper nor DSF alone has such potent effect (Fig. 1B, top). As previously observed (34), neither TM nor the TM-copper complex can inhibit the proteasomal chymotrypsin-like activity (Fig. 1B, top). Consistent with the inhibition of the proteasomal chymotrypsin-like activity, significantly increased levels of polyubiquitinated proteins were detected in the lysates prepared from the cells treated with the DSF-copper complex, compared with the control or DSF alone–treated cells (Fig. 1B, bottom). Copper alone, TM alone, or the TM-copper complex had some effect, but much less than that of the DSF-copper complex (Fig. 1B, bottom). Collectively, these results show that the DSF-copper complex potently inhibits the proteasome activity in intact breast cancer cells.

**The growth-inhibitory and apoptosis-inducing effects of the DSF-copper complex in breast cancer cells are associated with inhibition of cellular proteasomal chymotrypsin-like activity.** It has been shown that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of tumor cell growth arrest and/or apoptosis (23–25). To determine whether proteasome inhibition by the DSF-copper complex (Fig. 1B) causes suppression of cell proliferation, MDA-MB-231 cells were treated for 24 hours with copper, DSF, DSF-copper complex, TM, the TM-copper complex, or equal volume of the vehicle DMSO. We found that the DSF-copper complex inhibited proliferation of MDA-MB-231
cells in a concentration-dependent manner, by 55%, 65%, and 85%, respectively, when used at 5, 10 and 20 μmol/L (Fig. 1C). In contrast, all the other treatments at up to at 20 μmol/L had very little inhibitory effect on MDA-MB-231 cell proliferation (Fig. 1C).

To determine whether inhibition of growth is due to induction of apoptotic cell death, aliquots of the breast cancer MDA-MB-231 cells after each treatment (Fig. 1B) were subjected to Western blot analysis for measurement of PARP cleavage, an indicator of caspase activation and apoptosis induction (35). PARP cleavage was detected in the breast cancer cells treated with only the DSF-copper complex but not with others (Fig. 1B, bottom), showing that the DSF-copper complex induced apoptosis in breast cancer cells.

To further confirm the apoptosis-inducing ability of the DSF-copper complex, we measured apoptosis-associated cellular and nuclear morphologic changes in the same experiment. Cellular morphology changes (i.e., spherical and detached changes) were visualized by phase-contrast microscope imaging and apoptotic nuclear changes (i.e., punctuated or granular and bright nuclei) were determined after Hoechst dye staining. The apoptotic cellular (Fig. 1D, left) and nuclear changes (Fig. 1D, right) were observed only in the cells treated with the DSF-copper complex, but not with other agents or DMSO. These results further support the conclusion that the DSF-copper complex can induce apoptosis in breast cancer cells.

If proteasome inhibition is responsible for apoptosis induction by the DSF-copper mixture, we would expect that the proteasomal activity would be inhibited before the apoptotic events occur. To test this idea, we did a kinetic experiment in which MDA-MB-231 cells were treated with 15 μmol/L of CuCl₂, DSF, or DSF-copper for different hours. The results showed that proteasome inhibition by DSF-copper started at as early as 0.5 hour, because at this time point, the levels of proteasome activity were decreased by ~30% (Fig. 2A) and accumulation of ubiquitinated proteins was significantly increased (Fig. 2B). Importantly, cell death was not observed after 0.5 hour of treatment with DSF-copper, as shown by lack of PARP cleavage (Fig. 2B) and lack of cellular morphologic change (Fig. 2C). From 0.5 to 2 hours, proteasome activity was continuously inhibited while apoptosis had not started yet (Fig. 2). Apoptosis started at 6 hours in the cells treated with DSF-copper and further increased afterwards (Fig. 2B and C). As a comparison, neither CuCl₂ nor DSF alone was able to induce any of these events (Fig. 2). The results clearly show that the apoptosis induced by DSF-copper treatment is a consequential event of the proteasome inhibition.

The nontoxic effect of the DSF-copper complex in normal, immortalized breast cells. The ability to induce apoptosis in tumor, but not normal, cells is an important criterion for novel anticancer drugs. To determine whether the DSF-copper complex could inhibit the proteasome activity and induce apoptosis selectively in breast cancer over normal cells, we used a pair of normal and malignant breast cell lines, MCF-10A and MCF10DCIS.com. MCF-10A cells are normal, immortalized breast cells, whereas MCF10DCIS.com cells are malignant, which were derived from MCF-10A cells (33). Both MCF-10A and MCF10DCIS.com cell lines were treated with 20 μmol/L of copper, DSF, the DSF-copper complex, TM, the TM-copper complex, or DMSO for 24 hours, followed by measurement of proteasome inhibition and apoptosis. We found that the DSF-copper complex, but not others, strongly inhibited the proteasomal chymotrypsin-like activity in breast malignant MCF10DCIS.com cells (97%; Fig. 3A), similar to that observed in breast cancer MDA-MB-231 cells (Fig. 1B). In sharp contrast, when lysates of nontransformed MCF-10A cells treated with the DSF-copper complex were analyzed, no proteasome inhibition was detected (Fig. 3A). Other treatments had little or no effects on either MCF10DCIS.com or MCF-10A cells (Fig. 3A).

To determine whether failure of the DSF-copper complex to inhibit the proteasome activity in MCF-10A cells is associated with lack of apoptosis induction in these normal, immortalized breast cells, apoptosis-associated cellular and nuclear morphologic changes were then measured in the aliquots of both cell lines in the same experiment. The MCF10DCIS.com cells treated with the DSF-copper complex, but not others, were fully detached (Fig. 3B) and also showed the apoptotic nuclear changes (Fig. 3C). However, the normal, immortalized MCF-10A cells showed only little, if any, such cell death–related detachment or apoptotic nuclei after
treatment with the DSF-copper complex or others (Fig. 3B and C). Our data suggest that the DSF-copper complex could inhibit the proteasomal activity and induce apoptosis selectively in malignant cells but not in normal, immortalized breast cells.

The effects of DSF in copper-enriched breast cancer cells. Fundamental to the strategy we are using is the ability of the nontoxic ligand DSF to bind with endogenous tumor cellular copper. Cancer cells contain high level of copper in vivo (7–13). However, we found that the cultured cancer cells possess low to trace levels of copper (25, 34). To mimic the in vivo situation, human breast cancer MDA-MB-231 cells were cultured in medium containing 25 μmol/L CuCl₂ for 3 days. Cellular copper concentrations increased severalfold up to micromolar range (25, 34), similar to the copper concentrations found in patients (0.3-20 μmol/L; refs. 9, 10). Then the CuCl₂-precultured MDA-MB-231 cells were incubated with normal growth medium (without detectable copper) and treated with 20 μmol/L of DSF or TM for 24 hours, followed by measurement of proteasome inhibition and apoptosis.

The proteasomal chymotrypsin-like activity was inhibited by 67% in the cells treated with DSF compared with the control (Fig. 4A). In addition, accumulation of ubiquitinated proteins and cleavage of PARP were observed in the extract of the cells pretreated with CuCl₂ and posttreated with DSF (Fig. 4B). In contrast, when the copper-enriched cells were treated with TM, neither proteasome inhibition nor apoptosis induction was observed (Fig. 4). Therefore, DSF is able to induce proteasome inhibition and apoptosis in MDA-MB-231 cells containing increased copper levels (Fig. 4), but not in those with undetectable copper (Fig. 1).

The proteasome-inhibitory, apoptosis-inducing, and anti-tumor effects of DSF in human breast tumor xenografts. Our data described above clearly showed that the DSF-copper complex is a proteasome inhibitor and an apoptosis inducer in cultured human breast cancer (but not normal, immortalized) cells (Figs. 1-3) and that DSF alone can do so in breast cancer cells containing increased cellular copper (Fig. 4). It has been shown that treatment with the strong copper chelator TM inhibits growth of human breast, prostate, and lung tumors in various mouse models (36–38), indicating that human tumor xenografts in mice also contain high levels of copper. If so, DSF alone treatment should be able to induce proteasome inhibition and apoptosis in human tumor xenografts, leading to tumor growth inhibition. To test this idea, human breast cancer MDA-MB-231 cells were implanted s.c. to female nude mice. When tumors became palpable (~200 mm³), the mice were randomly grouped (total 10 mice per group from two experiments) and injected i.p. daily with either vehicle control or 50 mg/kg DSF. The injections were kept for 29 days until the control tumors reached ~1,600 mm³. During the

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**Figure 3.** The differential effects of DSF-copper complex in normal and malignant breast cells. Normal, immortalized human breast MCF-10A cells and malignant MCF10DCIS.com (DCIS) cells were treated with 20 μmol/L of copper, DSF, DSF-copper complex, TM, or TM-copper complex for 24 hours. DMSO was used as vehicle control. A, the inhibition of chymotrypsin-like activity was shown in MCF10DCIS.com cells, but not in normal MCF-10A cells treated with the DSF-copper complex. ++++, P < 0.001. Columns, mean of three experiments; bars, SD. B, cellular spherical and detached changes (indicating apoptosis) were observed only in malignant MCF10DCIS.com cells, but not in normal MCF-10A cells, after treatment with the DSF-copper complex. C, punctuated, granular, and brighter nuclei (apoptotic nuclei) were observed in MCF10DCIS.com cells, but not in normal MCF-10A cells, treated with the DSF-copper complex after staining with Hoechst 33258.

**Figure 4.** The proteasome-inhibitory and apoptosis-inducing effects of DSF in copper-enriched MDA-MB-231 cells. MDA-MB-231 cells were cultured in medium containing 25 μmol/L of copper for 3 days and then maintained in normal growth medium (without additional copper). This was followed by treatment with 20 μmol/L of DSF or TM or vehicle DMSO for 24 hours and preparation of cell extracts. A, the proteasomal chymotrypsin-like activity was inhibited only in the cells treated by DSF (++, P < 0.001). No proteasome inhibition was seen in TM-treated cells. Columns, mean of three experiments; bars, SD. B, Western blot analysis showed accumulation of ubiquitinated proteins and cleavage of PARP in cells treated with DSF, but not with TM.

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**Cu-Dependent Proteasome Inhibition by Disulfiram**

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treatment, the tumor sizes in these two groups were measured and shown in Fig. 5A (left). At the end of the experiment, the mice were sacrificed. The tumors were removed from the mice and photographed (Fig. 5A, right). We found that DSF significantly inhibited tumor growth by 74% ($P < 0.01$) compared with the solvent control (Fig. 5A). Therefore, the data show that DSF possesses potent antitumor effects in vivo.

To investigate whether the observed antitumor effects of DSF are associated with proteasome-inhibitory and apoptosis-inducing activities in vivo, samples of the control- or DSF-treated tumors were subjected to multiple assays. The proteasomal chymotrypsin-like activity was inhibited significantly (by 87%) in tumor tissues from mice treated with DSF when compared with the control (Fig. 5B), indicating that DSF inhibits the proteasomal activity in MDA-MB-231 tumors. Consistently, accumulation of ubiquitinated proteins and natural proteasome target proteins, p27 (39) and Bax (40, 41), was found in tumors treated with DSF versus control by Western blot analysis (Fig. 5D). Increased accumulation of p27 protein in tumors by DSF treatment was further confirmed by immunohistochemistry assay (Fig. 6A). Accompanying proteasome inhibition, apoptosis was induced in the MDA-MB-231 xenografts treated with DSF, as shown by the increased caspase-3 activity in tumor tissue extracts (Fig. 5C), the appearance of cleaved PARP fragment (Fig. 5D), increased terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells (Fig. 6B), and high levels of condensed apoptotic nuclei detected by H&E staining (Fig. 6C). The results clearly show that DSF was able to target the proteasome in vivo, resulting in accumulation of natural tumor suppressor proteins (such as p27 and Bax) and induction of apoptotic cell death within the tumor, which may be responsible for the observed potent antitumor activity of DSF (Fig. 5A).

**Discussion**

Many anticancer agents are unable to distinguish tumor from normal cells, which is perhaps responsible for their observed toxicity. Therefore, specific and selective targeting of chemotherapeutic drugs to the cancer, but not normal, cells could be of great benefit for cancer patients. Proteasome inhibition and antiangiogenesis 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/processes than normal cells (3–6, 19–21, 42–44). It is unique that copper, but not other trace metals in the body, is a cofactor essential and requisite for the tumor angiogenesis processes (3–6). Furthermore, it is well documented that cancer...
cells and tissues accumulate high levels of copper in vivo (7–13). Previously, we reported that certain types of organic-copper complexes are capable of proteasome inhibition and apoptosis induction in cancer cells (25, 34). Therefore, the capability of copper-containing complexes to inhibit the proteasome, the necessity of copper for angiogenesis, and the accumulation of copper by cancer cells and tissues allow for a novel therapeutic strategy focusing on elevated copper as a selective mechanism against cancer cells and tissues (3–6, 25, 34).

DSF has been approved by the Food and Drug Administration for the treatment of alcoholism in 1951 by inhibition of aldehyde dehydrogenase (28, 45). It was reported that DSF itself had little effect, but in the presence of Cu(II), it was converted back to the two-electron oxidized form of diethyldithiocarbamate, which is the active form in cancer cells (32). DSF is absorbed as its bis(diethyldithiocarbamato)-copper(II) complex (28), suggesting that the cells treated with the DSF-copper complex had significantly reduced chymotrypsin-like activity and increased ubiquitinated protein levels (Fig. 1D), indicating that proteasome inhibition had occurred. In contrast, copper, DSF, TM, or the TM-copper complex was incapable of inhibiting the proteasome (Fig. 1D). We also measured effects of these agents on MDA-MB-231 cell proliferation, PARP cleavage, and cellular and nuclear morphologic changes, and found that only the DSF-copper complex inhibited cell proliferation (Fig. 1C), induced cleavage of PARP (Fig. 1B), and caused cell death–associated morphologic and nuclear changes (Fig. 1D).

The following arguments support the idea that proteasome inhibition by DSF-copper is the cause, not the consequence, of apoptotic cell death. First, when MDA-MB-231 cells were treated with DSF-copper, the proteasome activity was inhibited at as early as 0.5 hour, about 5 hours before apoptosis induction (Fig. 2). Second, accumulation of tumor suppressor proteins p27 or Bax was also found in DSF-copper–treated tumors undergoing apoptosis (Figs. 5 and 6).

After determining that the DSF-copper complex could inhibit proteasome activity and cell proliferation and induce apoptosis in MDA-MB-231 cells (Fig. 1), we then examined the possibility that DSF-copper complex could specifically and selectively inhibit proteasome activity and induce apoptosis in breast cancer, but not normal, cells. Indeed, our results showed that the DSF-copper complex was a potent proteasome inhibitor and apoptosis inducer.

Figure 6. Immunohistochemistry, TUNEL, and H&E staining assays using mouse tumor samples. Tumors were collected after 29-day treatment (see Fig. 5 legend) and the prepared tissue slides were used for immunostaining with p27 antibody (A), TUNEL assay (B), and H&E staining assay (C). Stronger or/and more p27-positive cells and TUNEL-positive nuclei as well as apoptotic condensed nuclei were found in tumor tissue from mice treated with DSF. Magnification, ×400.
only in human breast cancer MCF10DCIS.com cells, but not in normal, immortalized human breast MCF10A cells (Fig. 3).

Many cancer tissues contain highly elevated levels of copper (7–13) although cultured tumors contain low or undetectable copper content (25, 34). To mimic the in vivo tumor environment, MDA-MB-231 cells were cultured in copper-enriched media for 3 days, then maintained in normal growth medium. This treatment caused a significant increase in cellular copper concentrations (up to 0.2-6 μmol/L range; refs. 25, 34), which were similar to the copper concentrations found in patients (0.3-20 μmol/L; refs. 9, 10). Afterwards, the copper-enriched MDA-MB-231 cells were treated with the ligand DSF or TM. The results support our hypothesis that the organic ligand DSF could interact with tumor cellular copper and form an active, specific proteasome-inhibitory complex, which leads to apoptosis induction (Fig. 4). Although the strong metal chelator TM should bind to copper in cancer cells, the resulted complex seems inactive in such functions (Fig. 4). Most recently, one group reported that DSF alone at ~0.16 μmol/L has proteasome-inhibitory activity under a cell-based screening assay condition (49). However, under our experimental conditions, DSF at 15 to 20 μmol/L was unable to inhibit cellular proteasome activity in cultured breast cancer cells (Figs. 1 and 2). This difference could be due to different cell systems, different levels of cellular copper, and/or different sensitivity of proteasome activity assays. Our study suggests that the proteasome-inhibitory activity of DSF observed under their conditions could be due to formation of an active complex between DSF and cellular copper.

To answer the question of whether DSF can react with copper in tumor tissue and possess antitumor activity, we tested effect of DSF in mice bearing human breast tumor MDA-MB-231 xenografts. Our data showed that DSF treatment caused a significant inhibition of MDA-MB-231 tumor growth in nude mice (Fig. 5A). Also importantly, the antitumor activity of DSF was associated with its proteasome-inhibitory and apoptosis-inducing abilities because DSF treatment resulted in the inhibition of proteasomal chymotrypsin-like activity in tumors (Fig. 5B), accumulation of proteasome target proteins p27 (Figs. 5D and 6A) and Bax (Fig. 5D), and induction of apoptosis (i.e., increase in caspase-3/caspase-7 activity, PARP cleavage, TUNEL positivity, and condensed nuclei; Figs. 5 and 6). A previous study has suggested the antitumor activity of DSF in melanoma and hepatic tumor, which could be potentiated by Zn2+ supplementation (46). We have found that a DSF-zinc complex is also a proteasome inhibitor although its potency is weaker than that of the DSF-copper complex.1 Our results presented here have further confirmed their finding and also showed the requirement of proteasome inhibition for the antitumor activity of DSF.

The data presented here support the novel concept of using accumulated copper in breast cancer cells and tissues as a selective approach for chemotherapy. The nontoxic copper-binding ligands such as DSF can spontaneously bind with copper and form a proteasome inhibitor and an apoptosis inducer in breast cancer, but not normal, cells. Cancer cells and tissues, which contain elevated copper and more dependent on proteasome activity for their survival, should be very sensitive to treatment with DSF and other copper-binding compounds. In contrast, normal cells and tissues, containing trace amounts of copper and having basal level of proteasome activity, should be resistant to these effects. DSF has been previously explored for use in clinic for alcoholism, and our data suggest the potential use of DSF and other similar compounds in cancer therapies.

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1 Unpublished data.
Cu-Dependent Proteasome Inhibition by Disulfiram


Disulfiram, a Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts via Inhibition of the Proteasome Activity

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