A Novel Anticancer Gold(III) Dithiocarbamate Compound Inhibits the Activity of a Purified 20S Proteasome and 26S Proteasome in Human Breast Cancer Cell Cultures and Xenografts

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Abstract
Although cisplatin has been used for decades to treat human cancer, some toxic side effects and resistance are observed. It has been suggested that gold(III) complexes, containing metal centers isoelectronic and isostructural to cisplatin, are promising anticancer drugs. Gold(III) dithiocarbamate complexes were shown to exhibit in vitro cytotoxicity, comparable with and even greater than cisplatin; however, the involved mechanism of action remained unknown. Because we previously reported that copper(II) dithiocarbamates are potent proteasome inhibitors, we hypothesized that gold(III) dithiocarbamate complexes could suppress tumor growth via direct inhibition of the proteasome activity. Here, for the first time, we report that a synthetic gold(III) dithiocarbamate (compound 2) potently inhibits the activity of a purified rabbit 20S proteasome and 26S proteasome in intact highly metastatic MDA-MB-231 breast cancer cells, resulting in the accumulation of ubiquitinated proteins and the proteasome target protein p27 and induction of apoptosis. The compound 2–mediated proteasome inhibition and apoptosis induction were completely blocked by addition of a reducing agent DTT or N-acetyl-L-cysteine, showing that process of oxidation is required for proteasome inhibition by compound 2. Treatment of MDA-MB-231 breast tumor–bearing nude mice with compound 2 resulted in significant inhibition of tumor growth, associated with proteasome inhibition and massive apoptosis induction in vivo. Our findings reveal the proteasome as a primary target for gold(III) dithiocarbamates and support the idea for their potential use as anticancer therapeutics. (Cancer Res 2006; 66(21): 10478-86)

Introduction
Metal compounds have been used for treatment of various diseases for centuries, although the molecular mechanism of their activity was not fully understood. Half a century ago, it was observed that metal ions were capable of binding to nucleic acids, thereby altering their conformation and biological function (1). This finding opened a new, promising avenue for cancer treatment using metal-based drugs. After successes achieved with platinum complexes, such as cisplatin, against selected types of cancers (2), the search for other metals that might produce more specific anticancer effects continued. This search was further driven by observations that the use of cisplatin in curative therapy was associated with some serious clinical problems, such as severe normal tissue toxicity and resistance to the treatment (3).

Because of their anti-inflammatory and immunosuppressive properties, some gold(I) compounds used for the treatment of rheumatoid arthritis (4), such as gold thiolates, were considered for their possible anticancer activity (5). It has been shown that gold(I) compounds inhibit tumor cell proliferation in vitro (6), but unfortunately, their in vivo effectiveness was found to be very limited (7). Because gold(III) is isoelectronic with platinum(II), and because tetracoordinate gold(III) complexes are in the same square-planar geometries as cisplatin (8), the anticancer activity of gold(III) compounds has been investigated.

Recently, new, stable gold(III) compounds have been synthesized by using ligands that usually have nitrogen atoms as donor groups (9). Studies of these gold(III) complexes showed that their interactions with DNA, the classic target of platinum(II) complexes, are not as tight as platinum drugs, suggesting a different cytotoxic mechanism (10, 11). This observation has prompted a new search for gold-protein interactions in an attempt to identify possible targets responsible for the biological effects of gold compounds. Another recent report showed that in an in vitro system several new gold(III) dithiocarbamate derivatives were cytotoxic, comparable with, and even greater than, cisplatin toward a series of human tumor cell lines (2). They act fast, inhibit DNA and RNA synthesis, and show only a minimal cross-resistance with cisplatin (8), suggesting a different mechanism of action.

The ubiquitin-proteasome pathway is essential for many fundamental cellular processes, including the cell cycle, apoptosis, angiogenesis, and differentiation (12). The proteasome contributes to the pathologic 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 (13). The 20S proteasome, the proteolytic core of 26S proteasome complex, contains multiple peptidase activities [chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydroylizing-like (PGPH)] and functions as a catalytic machine (14).

The possibility of targeting the ubiquitin-proteasome pathway therapeutically was met with great skepticism at the very beginning because this pathway plays an important role in normal cellular homeostasis as well. However, with the demonstration that proteasome inhibitors were well tolerated and had activity in models of human malignancies in vivo (15), the proteasome inhibitor Velcade/PS-341 was introduced into phase I safety trials (16). The data from the Velcade trials showed acceptable toxicity with significant clinical benefit (17). Furthermore, the fact that...
actively proliferating cancer cells are more sensitive to apoptosis-inducing stimuli, including proteasome inhibitors, makes proteasome inhibitors even more attractive (18–20).

We have previously shown that some metals, such as copper, can inhibit the proteasome (21, 22). We, therefore, hypothesized that gold(III) dithiocarbamate complexes suppress tumor growth via direct inhibition of the proteasome activity. In this study, we show for the first time that the gold(III) dithiocarbamate derivative (compound 2) inhibits the chymotrypsin-like activity of a purified rabbit 20S proteasome (IC50 = 7.4 μmol/L) and the 26S proteasome in intact highly metastatic MDA-MB-231 breast cancer cells (10-20 μmol/L). Inhibition of the proteasome activity results in the accumulation of ubiquitinated proteins and the proteasome target protein p27 and induction of apoptosis. Addition of a reducing agent completely blocks compound 2–induced proteasome inhibition and apoptosis induction. Treatment of breast cancer–bearing nude mice with compound 2 resulted in tumor growth inhibition and massive apoptosis induction, associated with proteasome inhibition in vivo.

Materials and Methods

Materials. Synthetic gold(III) dithiocarbamate derivatives (compounds 1-4), inorganic gold salts (KAuBr4 and KAuCl4), and ligands [XV-dimethyl-dithiocarbamate (DMDT) and S-methyl-ethylsarcosinedithiocarbamate (ESDTM)] were described previously (2, 8). Biebenzimide Hoechst No. 33258 stain, cholera toxin, hydrocortisone, 3-[4,5-dimethyltiazol-2-yl]-2.5-diphenyl-tetrazolium bromide (MTT), epidermal growth factor, insulin, CuCl2, DMSO, DTT, N-acetyl-cysteine (NAC), cromophor, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, horse serum, penicillin, and streptomyocin were purchased from Invitrogen (Carlsbad, CA). Purified rabbit 20S proteasome and fluorescent peptide substrates Suc-LYV-AMC, Z-LE-AMC, and Z-ARR-AMC (for the proteasomal chymotrypsin-like, PGH-like, trypsin-like activities, respectively) were from Calbiochem (San Diego, CA). 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), and secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture and cell extract preparation. MCF10AT1K.cl2 and MCF10kcis.com cells were cultured as previously described (22). MCF7 cells were grown in MEME supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomyocin, and 0.4% of bovine insulin. MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomyocin. All cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO2. A whole-cell extract was prepared as previously described (23).

Analysis of the proteasomal activity in purified 20S proteasome and whole-cell extract. Purified rabbit 20S proteasome (35 ng) or MDA-MB-231 whole-cell extract (10 μg) was incubated with 20 μmol/L of the various substrates in 100 μl of assay buffer [20 μmol/L Tris- HCl (pH 7.5)] in the presence of different synthetic gold compounds, inorganic gold salts, and ligands at various concentrations or equivalent volume of solvent DMSO as control. After 2 hours of incubation at 37°C, inhibition of each proteasomal activity was measured (24–26).

Proteasome activity assay in intact breast cancer MDA-MB-231 cells. MDA-MB-231 breast cancer cells were grown to 70% to 80% confluency, treated under various conditions, harvested, and used for whole-cell extract preparation. Ten micrograms of cell extract was used to determine the chymotrypsin-like activity, as described above.

Cell proliferation assay. The MTT assay was used to determine the effects of various compounds on breast cancer cell proliferation. Cells were plated in a 96-well plate and grown to 70% to 80% confluency followed by addition of each compound at the indicated concentrations. After 24 hours of incubation at 37°C, inhibition of cell proliferation was measured as previously described (22).

Cellular and nuclear morphology analysis. A whole-cell extract was prepared as previously described (22).

Western blot analysis. MDA-MB-231 cells were treated, harvested, and lysed. 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 (26).

Human breast tumor xenograft experiments. Female athymic nude mice ages 5 weeks were purchased from Taconic Research Animal Services (Hudson, NY) and housed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. Human breast cancer MDA-MB-231 cells (5 × 106) suspended in 0.1 ml of serum-free RPMI 1640 were inoculated s.c. in both flanks of each mouse (four mice per group). When tumors reached sizes of ~150 mm3, the mice were randomly grouped and treated by daily s.c. injection with either 1.0 or 2.0 mg/kg of compound 2 or vehicle [20% DMSO and 80% Cremophore/ethanol (3:1)]. Tumor size was measured every other day using calipers, and their volumes were calculated according to a standard formula: (width2 × length) / 2. Mice were sacrificed after 29 days of treatment when control tumors reached ~1,300 mm3. The tumors were collected and photographed, and the tumor tissues were used for different assays for measuring proteasome inhibition and cell death.

Results

The synthetic gold(III) dithiocarbamate compound 2 inhibits proliferation of various breast cancer cell lines. Recently, four different gold(III) dithiocarbamate derivatives (compounds 1-4) were reported to induce cell death in several human tumor cell lines (2). These compounds were also shown to interact with DNA in a weak and reversible manner, suggesting that DNA is not their primary target (8). In the current study, we investigated the effect of these gold compounds on human breast cancer cells and, more importantly, the molecular mechanism responsible for their tumor cell–killing activity and their potential antitumor activity.

We selected compound 2 that was synthesized by a direct reaction of the ligand DMDT with the gold salt KAuBr4 (Fig. 1A; ref. 8). We also prepared a DMDT-KAuBr4 mixture by mixing both at a 1:1 ratio, which resulted in a color change (data not shown), indicating a chemical reaction and metal complex formation. We first tested the growth-inhibitory effect of compound 2 on several breast cancer cell lines, including premalignant MCF10AT1K.cl2, malignant MCF10kcis.com, estrogen receptor α–positive MCF-7, and estrogen receptor α–negative MDA-MB-231. Compound 2 (at as low as 5 μmol/L) inhibited the proliferation of all these tested breast cancer cell lines (Fig. 1B). When used at 20 μmol/L, compound 2 inhibited growth in about 80% of premalignant MCF10AT1K.cl2 cells and about 90% of the other tested breast cancer cell lines (Fig. 1B). Because MDA-MB-231 cells are highly...
metastatic and invasive, we decided to continue our investigation with this breast cancer cell line.

To confirm the proliferation-inhibitory effect of compound 2, MDA-MB-231 cells were treated with 10 or 20 μmol/L of the ligand DMDT alone, gold salt KAuBr₄ alone, the DMDT-KAuBr₄ mixture, or synthetic compound 2 followed by an MTT assay. The DMDT-KAuBr₄ mixture and compound 2 at 10 μmol/L inhibited growth of MDA-MB-231 cells by ~70% and ~85%, respectively (Fig. 1C). Treatment with 20 μmol/L of DMDT-KAuBr₄ mixture or compound 2 inhibited about 90% of cell proliferation (Fig. 1C). In contrast, neither the ligand DMDT nor gold salt KAuBr₄ alone had much effect (Fig. 1C). As a comparison, a DMDT-CuCl₂ mixture at both concentrations inhibited ~90% of cell proliferation, whereas copper salt alone had no effect (Fig. 1C).

Inhibition of the proteasome activities by gold(III) salt and synthetic gold(III) compounds in vitro. We have shown that some organic copper compounds can inhibit proteasome activity (21, 22) and therefore hypothesized that gold(III) compounds could likewise target the tumor cellular proteasome. To test this hypothesis, we first measured the effect of four gold(III) compounds on the proteasomal chymotrypsin-like activity of MDA-MB-231 cell protein extract. All the tested synthetic gold(III) complexes (compounds 1-4) potently inhibited the proteasomal chymotrypsin-like activity in a concentration-dependent manner (Fig. 2A). In addition, both gold salts (KAuCl₄ and KAuBr₄) alone were able to inhibit the proteasomal chymotrypsin-like activity with potency weaker than that of synthetic gold complexes or CuCl₂ (Fig. 2A). In contrast, neither of the two ligands (DMDT and ESDTM) exhibited proteasome-inhibitory properties, even at high concentrations (Fig. 2A).

To provide direct evidence for proteasome inhibition by gold salts and synthetic gold(III) compound 2, we incubated a purified rabbit 20S proteasome with KAuBr₄ and compound 2 at various concentrations followed by measurement of various proteasomal activities. All the three activities (chymotrypsin-like, trypsin-like, and PGPH-like) of the purified 20S proteasome were significantly inhibited by synthetic gold compound 2 with similar potencies (IC₅₀ = 7.4, 10.2, and 7.0 μmol/L, respectively; Table 1). However, mainly the trypsin-like activity of purified 20S proteasome was inhibited by the inorganic gold salt KAuBr₄ (IC₅₀ = 1.2 μmol/L for trypsin-like versus IC₅₀ = 34.7 and 15.7 μmol/L for chymotrypsin-like and PGPH-like, respectively; Table 1). Therefore, synthesis of...
the organic complex (compound 2) from the inorganic gold(III) salt KAuBr₄ resulted in an increased selectivity to inhibit the proteasomal chymotrypsin-like activity.

Inhibition of cellular proteasomal activity by synthetic gold compound 2 is associated with apoptosis in MDA-MB-231 breast cancer cells. It has been shown that inhibition of the proteasomal chymotrypsin-like but not trypsin-like activity is associated with apoptosis induction in cancer cells (23, 28). To explore whether compound 2 has greater proteasomal chymotrypsin-like-inhibitory and apoptosis-inducing activities than the inorganic gold(III) salt, MDA-MB-231 cells were treated with 10 or 20 μmol/L of indicated compounds and mixtures (for 4 and 24 hours), collected, and analyzed for the chymotrypsin-like activity, as described in Materials and Methods.

Figure 2. Inhibition of proteasomal chymotrypsin-like activity and induction of apoptosis in MDA-MB-231 breast cancer cells by gold compounds. A, inhibition of proteasomal chymotrypsin-like activity in MDA-MB-231 cell extract. MDA-MB-231 cell extract (10 μg per reaction) was incubated with a peptide substrate for the proteasomal chymotrypsin-like activity in the presence of different compounds including gold salts KAuBr₄ (AuBr) and KAuCl₄ (AuCl), dithiocarbamate ligands (DMDT and ESDTM), and synthetic gold complexes (Comp 1–4) at the indicated concentrations. Copper salt (CuCl₂) was used as a positive control. B, inhibition of proteasomal chymotrypsin-like activity in intact MDA-MB-231 cells. The cells were treated with 20 μmol/L of indicated compounds and mixtures (for 4 and 24 hours), collected, and analyzed for the chymotrypsin-like activity, as described in Materials and Methods. C, Western analysis for accumulation of ubiquitinated proteins p27, Bax/p36, and PARP cleavage in the above prepared cell extracts.

Table 1. IC₅₀ values (μmol/L ± SD) for the 20S proteasomal activities of compound 2 and inorganic gold salt KAuBr₄

<table>
<thead>
<tr>
<th>Proteasomal activity</th>
<th>Compound 2</th>
<th>KAuBr₄</th>
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<tbody>
<tr>
<td>Chymotrypsin-like</td>
<td>7.4 ± 0.9</td>
<td>34.7 ± 0.3</td>
</tr>
<tr>
<td>Trypsin-like</td>
<td>10.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>PGPH-like</td>
<td>7.0 ± 0.4</td>
<td>15.7 ± 0.1</td>
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activity and accumulated levels of ubiquitinated proteins and p27 protein (Fig. 2B and C).

To investigate whether the proteasomal inhibition is associated with apoptosis induction, both morphologic changes and apoptosis-associated PARP cleavage were studied. Changes in cell morphology (shrunken cells and characteristic apoptotic blebbing) were observed after 4 hours only in the cells treated with compound 2 and the mixtures with either gold or copper salt, but not others (data not shown), and these changes were greatly increased after 24 hours of treatment (Fig. 3A). We noticed that morphologic changes in the cells treated with compound 2 and the DMDT-KAuBr₄ mixture started earlier than in the cells treated with the DMDT-CuCl₂ mixture. Consistent with apoptosis induction, we observed the presence of apoptotic nuclei in the cells treated for 24 hours with the synthetic compound 2 and both gold(III) and copper(II) mixtures after Hoescht staining (Fig. 3B). Furthermore, treatment with the DMDT-KAuBr₄ mixture and compound 2 as well as the DMDT-CuCl₂ mixture caused the disappearance of the intact PARP protein (116 kDa), associated with production of two fragments p85 and p65 (Fig. 2C). It has been shown that caspase-3/caspase-7 cleaves PARP, generating the p85 PARP fragment (29), and that calpain cleaves PARP and produces a fragment of ~65 kDa (30).

We and others have shown that associated with the apoptotic commitment, Bax protein (p21/Bax) could be cleaved by calpain, producing a p18/Bax fragment, which then forms a homodimer p36/Bax (31, 32). If gold(III) compounds can activate calpain and produce p65/PARP fragment (Fig. 2C; PARP), we would then expect the appearance of the Bax/p36 homodimer after treatment. Under our experimental conditions and in the untreated MDA-MB-231 cells, we detected three forms of Bax protein: p21, p18, and p36 (Fig. 2C; p18 and p36 could be from spontaneously apoptotic cells). Treatment with the DMDT-KAuBr₄ mixture or compound 2 as well as the DMDT-CuCl₂ mixture, but not others, caused the complete disappearance of p21/Bax and p18/Bax and the increased levels of p36/Bax (Fig. 2C). These results show that compound 2 is able to inhibit the proteasomal chymotrypsin-like activity, resulting in calpain activation and apoptosis induction in human breast cancer MDA-MB-231 cells.

Proteasome inhibition and apoptosis induction by compound 2 is blocked by the reducing agent NAC. It has been shown that gold and other metal salts can oxidize some cellular proteins, and that this oxidation can be blocked by the addition of reducing agents, such as NAC (33–36). Because the synthetic gold(III) compound 2 is able to inhibit the activity of purified rabbit 20S proteasome (Table 1) and breast cancer cellular proteasome (Fig. 2), we then tested the effect of a reducing agent, such as DTT or NAC, on compound 2–mediated events. We found that inhibition of purified 20S proteasome by compound 2, inorganic gold salt (KAuBr₄), or copper salt (CuCl₂) could be completely blocked by the reducing agent NAC.
reversed by 1 mmol/L DTT (Fig. 4A versus Table 1; ref. 21). Furthermore, when a whole-cell lysate was used, compound 2–induced proteasome inhibition was also inhibited by DTT (data not shown) and NAC (Fig. 4B) in a dose-dependent manner.

The effect of NAC on compound 2–induced proteasome inhibition in intact MDA-MB-231 cells was then investigated. We found that NAC at as low as 200 μmol/L was able to completely block the proteasome inhibition induced by compound 2 at both early (4 hours; data not shown) and later time points (24 hours; Fig. 4C). Importantly, NAC cotreatment also completely blocked compound 2–induced apoptotic morphologic changes (Fig. 3C) and production of p36/Bax induced by compound 2 is inhibited by NAC in the MDA-MB-231 cells. MDA-MB-231 cells were treated with 20 μmol/L of compound 2 for 24 hours in the absence or presence of NAC at indicated concentrations, followed by measuring proteasomal chymotrypsin (CT)–like activity (C) and Western blotting using specific antibodies to PARP, Bax, and β-actin (D).

Synthetic gold(III) compound 2 inhibits the growth of human breast cancer xenografts, associated with proteasome inhibition and apoptosis induction in vivo. To do so, we implanted MDA-MB-231 cells (5 × 10⁶) s.c. in 5-week-old female athymic nude mice. When the tumors were palpable (~150 mm³), the mice were then s.c. treated daily with either vehicle control or compound 2 at 1.0 mg/kg. Significant inhibition of tumor growth by compound 2 was observed after a 29-day treatment, showing that compound 2 has antitumor activity (Fig. 5A). Control tumors grew to an average size of 1,302 ± 71 mm³, and compound 2–treated tumors grew to 658 ± 12 mm³, corresponding to 50% inhibition (P < 0.01; Fig. 5A). The collected MDA-MB-231 tumors were then used for proteasome activity and apoptosis assays. The proteasomal chymotrypsin-like activity was inhibited by 40% in the tumors treated with compound 2 compared with control (Fig. 5B). Consistently, Western blot analysis showed that p27 protein was accumulated in tumors treated with compound 2 but not the control vehicle (Fig. 5C). With the same anti-p27 antibody, we also detected a very strong band of ~70 kDa only in the extracts of compound 2–treated tumors (Fig. 5C). According to the previous reports, this p70 band should be an ubiquitinated form of p27 (23, 37). In addition, immunostaining confirmed the increased expression of p27 only in the tumors treated with compound 2 (Fig. 5D). Therefore, compound 2 is able to inhibit tumor proteasome activity in vivo.

To determine whether apoptosis is responsible for the observed antitumor activity of compound 2 (Fig. 5A), several assays were...
done using tumor tissue samples. Apoptotic cells indicated by TUNEL positivity were observed in tumors from animals treated with compound 2 but not control (Fig. 5D). Another apoptotic feature (condensed nuclei) was shown by H&E staining in compound 2–treated tumors (Fig. 5D). Finally, a cell death–associated PARP cleavage fragment was detected in extracts of tumors treated with compound 2 compared with the control (Fig. 5C). In another animal experiment, when compound 2 was used at 2 mg/kg/d, we also observed similar antitumor, proteasome-inhibitory, and apoptotic effects (data not shown). Taken together, these data show that the ability of compound 2 to inhibit the proteasomal chymotrypsin-like activity and induce apoptosis in breast tumors in vivo is most probably responsible for its antitumor activity.

**Discussion**

Empirical data collected in the last 10 years suggested that metal-based compounds greatly varied with regard to their biological properties and the mechanisms underlying their antitumor activity. Although the mechanism of cisplatin is well understood, the mechanism responsible for gold(III) complex–induced cytotoxicity remained unclear. It has been suggested by Fricker et al. (35) that proteins, rather than DNA, might be the main target for gold(III) complexes. They found that a gold(III)–damp complex showed a clear preference for S-donor ligands, such as glutathione and cysteine, with only limited reactivity against nucleosides and their bases (35). Therefore, a new mechanism that proteins containing exposed cysteine residues might be proper targets for that class of gold(III) complexes was proposed. Gold(III) complexes were also shown to interact with bovine serum albumin (38), making very stable adducts that, once formed, may be destroyed only by the addition of strong ligands for gold(III), such as cyanide (39). Based on that, it has been proposed that selective modification of surface protein residues by gold(III) compounds could be the molecular basis for their biological effects. However, the key proteins that are modified by gold(III) complexes responsible for triggering apoptosis were not identified.

In this study, we showed that the gold(III) compound 2 potently inhibited proliferation of different breast cancer cell lines, including premalignant MCF10C2, malignant MCF10C2ics.com, estrogen receptor α–positive MCF7, and estrogen receptor α–negative MDA-MB-231 (Fig. 1B). In addition, compound 2 was much more potent than cisplatin under our experimental conditions.
conditions. Compound 2 at 5 μmol/L inhibited 85% of MDA-MB-231 cell proliferation compared with <20% inhibition by 5 μmol/L of cisplatin (data not shown). Even when 50 μmol/L of cisplatin was used, only ~40% inhibition was observed (data not shown). Moreover, compound 2 at 5 μmol/L for 2 hours induced apoptotic morphologic changes in MDA-MB-231 cells, whereas cisplatin at 50 μmol/L for 48 hours did not induce such changes (data not shown). Our data support the argument that the mechanisms of action of platinum(II) and gold(III) complexes are different.

To investigate the molecular mechanism responsible for the gold(III) complex–mediated tumor cell–killing activity, we used the highly metastatic and invasive MDA-MB-231 breast cancer cell line. We have previously shown that copper was capable of irreversible inhibition of the proteasome in a time- and concentration-dependent manner under in vitro conditions (21, 22). Therefore, we hypothesized that gold and copper might use the same mechanism against cancer cells. We found that all four tested gold(III) complexes (compounds 1-4) inhibited the proteasomal chymotrypsin-like activity in MDA-MB-231 whole-cell extract in a concentration-dependent manner (Fig. 2A). To provide direct evidence for proteasome inhibition by gold compounds, we did a cell-free proteasome activity assay using a purified 20S proteasome and compound 2 or the inorganic KAuBr4 salt. Interestingly, we discovered that compound 2 significantly inhibited all three activities of the purified 20S proteasome, whereas inorganic gold salt KAuBr4 inhibited mostly its trypsin-like activity (Table 1). It should be emphasized that synthesis of inorganic gold(III) salt into an organic complex greatly increased its preference of inhibiting the proteasomal chymotrypsin-like over the trypsin-like activity. This finding is particularly important because it has been reported that inhibition of proteasomal chymotrypsin-like but not trypsin-like activity is associated with growth arrest and/or apoptosis induction in cancer cells (23, 28).

After we showed that compound 2 could inhibit the purified proteasomal chymotrypsin-like activity, we then tested its effect in intact MDA-MB-231 cells and found similar inhibitory effects. Proteasomal inhibition by compound 2 was confirmed by decreased proteasomal activity (Fig. 2B) and increased levels of ubiquitinated proteins and the proteasome target protein p27 (Fig. 2C). Most importantly, inhibition of the proteasome activity and accumulation of p27 were also found in MDA-MB-231 xenografts treated with compound 2 (Fig. 5B-D). All together, these findings clearly indicate that compound 2 can directly target the tumor proteasome in vivo.

It has been reported that various proteasome inhibitors potently induce apoptosis (18, 19, 23, 28, 40). Therefore, we investigated if gold(III) compound 2 behaved similarly. Indeed, we found that inhibition of the proteasomal chymotrypsin-like activity by compound 2 induced apoptosis in cultured MDA-MB-231 breast cancer cells (Figs. 2 and 3) and in the tumors developed from the same breast cancer cell line (Fig. 5). Induction of apoptosis by compound 2 in vitro and in vivo has been shown by multiple assays that measure characteristic cellular and biochemical hallmarks. For instance, apoptotic morphologic changes (Fig. 3f), the presence of apoptotic nuclei (Fig. 3f), and apoptosis-specific PARP cleavage (Fig. 2C) were observed in cultured MDA-MB-231 cells treated with compound 2. In the treated tumors, apoptosis induction was confirmed by PARP cleavage (Fig. 5C), TUNEL, and H&E staining assays (Fig. 5D).

In this study, our data strongly suggest that the primary target for gold(III) dithiocarbamates is the proteasome, and that inhibition of the proteasomal activity by gold(III) dithiocarbamates is associated with apoptotic cancer cell death. We found that the effect of compound 2 could be completely blocked by two different S-donor ligands (DTT and NAC). DTT at 1 mmol/L entirely reversed inhibition of purified 20S proteasome by compound 2 and inorganic gold salt (Fig. 4A), and NAC at 200 μmol/L completely blocked proteasome inhibition by compound 2 in intact MDA-MB-231 cells at both early (4 hours) and later (24 hours) time points (Fig. 4C). Consistently, apoptotic morphologic changes (Fig. 3C), PARP cleavage (Fig. 4D), and accumulation of p36/Bax were also completely prevented in the cells cotreated with compound 2 and NAC. It should be noted that NAC at lower concentrations was unable to inhibit copper-mediated proteasome inhibition in vitro and in tumor cells (21). We are currently investigating whether NAC at higher concentration could reverse organic copper–induced events.

There are several possible mechanisms that might be responsible for the reversal of compound 2–mediated proteasomal inhibition by NAC and DTT. First, it has been previously reported that some gold(III) complexes could bind some S-donor ligands, such as glutathione and cysteine, and cleave their disulfide bond(s) (36), which might be responsible for the biological effects of gold(III) complexes (39). It is possible that gold(III) dithiocarbamates could bind NAC or DTT. NAC or DTT at high concentrations could react with all compound 2 (or gold salt) molecules, thereby preventing it from binding and inhibiting the proteasome. In the second possible mechanism, NAC or DTT could reduce gold(III) to gold(I), an ionic state that does not have the affinity to bind the proteasome and inhibit its activity. Third, it has been reported that gold(III) porphyrin 1a induces intracellular oxidation, altering reduced glutathione (GSH) levels in the cell (41). GSH is the main antioxidant system in the cell, and its depletion might facilitate accumulation of reactive oxygen species (ROS) in cells treated with anticancer drugs, which in turn increases the drug lethality (42). Compound 2 might stimulate production of ROS, which then oxidize and inactivate the proteasome. This argument is supported by a report that the proteasome is susceptible to oxidative modification and inactivation upon exposure to free radical–generating systems (43). Moreover, we observed that the effect of NAC is much stronger in the intact cells compared with the cell-free conditions (Fig. 4). When intact cells were treated with compound 2, NAC at much lower concentrations could reverse proteasomal inhibition induced by compound 2 (Fig. 4), arguing that NAC can increase cellular pool of ROS scavengers. We are currently investigating all these possibilities.

In summary, we identified the proteasome as a primary target for gold(III) dithiocarbamates in vitro and in vivo. We showed that the inhibition of the proteasomal activity (especially, chymotrypsin-like activity) by compound 2 is a strong apoptotic stimulus in the highly metastatic MDA-MB-231 breast cancer cell cultures and tumors. When proteasomal inhibition in cultured cells was blocked by S-donor ligands and reducing agents NAC and DTT, apoptosis was prevented. We also showed that s.c. treatment of MDA-MB-231 tumor-bearing nude mice with compound 2 resulted in significant inhibition of tumor growth, as a consequence of proteasomal inhibition and apoptosis induction. During the 29-day treatment with compound 2 at 1 to 2 mg/kg/d, no toxicity was observed, and mice did not display signs of weight loss, decreased activity, or anorexia. However, more detailed...
microscopic and macroscopic pathologic studies are required to confirm the lack of toxicity under these conditions. It has been known that other metal-based drugs that target DNA, such as cisplatin, are often associated with the occurrence of secondary malignancies over the course of treatment. Therefore, our finding that the proteasome is targeted by the gold complexes is particularly important with regard to development of novel anticancer drugs.

References


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References

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