ARTICLE
Journal of Cellular Biochemistry 109:162–172 (2010)

Journal of Cellular Biochemistry

Inhibition of Tumor Proteasome Activity by Gold–Dithiocarbamato Complexes via Both Redox-Dependent and -Independent Processes

Xia Zhang,[1,2] Michael Frezza,[1] Vesna Milacic,[1] Luca Ronconi,[3] Yuhua Fan,[2] Caifeng Bi,[2] Dolores Fregona,[3] and Q. Ping Dou[1*]

[1] *The Prevention Program, Department of Pathology, Barbara Ann Karmanos Cancer Institute, School of Medicine, Wayne State University, Detroit, Michigan*

[2] *Key Laboratory of Marine Chemistry Engineering and Technology, Ministry of Education, College of Chemistry and Chemical Engineering, Ocean University of China, Qingdao 266100, People's Republic of China*

[3] *Department of Chemical Sciences, University of Padova, Via Marzolo 1, 35131 Padova, Italy*

ABSTRACT

We have previously reported on a gold(III) complex, namely [$AuBr_2$(DMDT)] (*N*,*N*-dimethyldithiocarbamate) showing potent in vitro and in vivo growth inhibitory activities toward human cancer cells and identifying the cellular proteasome as one of the major targets. However, the importance of the oxidation state of the gold center and the involved mechanism of action has yet to be established. Here we show that both gold(III)– and gold(I)–dithiocarbamato species, namely [$AuBr_2$(ESDT)] (AUL12) and [Au(ESDT)]$_2$ (AUL15), could inhibit the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in human breast cancer MDA-MB-231 cells, resulting in accumulation of ubiquitinated proteins and proteasome target proteins, and induction of cell death, but at significantly different levels. Gold(I)- and gold(III)-compound-mediated proteasome inhibition and cell death induction were completely reversed by the addition of a reducing agent, dithiothreitol or *N*-acetyl-L-cysteine, suggesting the involvement of redox processes. Furthermore, treatment of MDA-MB-231 cells with gold(III) compound (AUL12), but not the gold(I) analog (AUL15), resulted in the production of significant levels of reactive oxygen species. Our study provides strong evidence that the cellular proteasome is an important target of both gold(I) and gold(III)–dithiocarbamates, but distinct cellular mechanisms of action are responsible for their different overall effect. J. Cell. Biochem. 109: 162–172, 2010.

KEY WORDS: APOPTOSIS; GOLD COMPOUNDS; PROTEASOME INHIBITOR; REDOX; REACTIVE OXYGEN SPECIES

The medicinal applications of metal ions have been recognized in the treatment of various diseases, including cancer. The platinum-based drug cisplatin is one of the most widely employed anti-cancer agents, exhibiting clinical effectiveness in several malignancies, including a curative effect in >90% of testicular cancers [Wong and Giandomenico, 1999]. To avoid or reduce serious side effects observed in some cisplatin-treated patients, numerous platinum-based analogs were synthesized and evaluated. Although many of them exhibited significant clinical benefit, their widespread use has still been hampered by tumor drug resistance and non-specific toxicity [Criado et al., 2003].

Abbreviations used: AUL12, [$AuBr_2$(ESDT)]; AUL15, [Au(ESDT)]$_2$; CT-like, chymotrypsin-like; DTT, dithiothreitol; DMDT, *N*,*N*-dimethyldithiocarbamate, $(CH_3)_2NCSS^-$; DMSO, dimethylsulfoxide; ESDT, ethylsarcosinedithiocarbamate, $CH_3CH_2OC(O)CH_2N(CH_3)CSS^-$; MTT, 3-(4,5-dimthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; PARP, poly(ADP-ribose)polymerase; ROS, reactive oxygen species; TBHP, *t*-butylhydroperoxide.

Xia Zhang and Michael Frezza contributed equally to this study.

The authors disclose no conflicts of interest.

Grant sponsor: Karmanos Cancer Institute of Wayne State University; Grant sponsor: Department of Defense Breast Cancer Research Program Awards; Grant numbers: W81XWH-04-1-0688, DAMD17-03-1-0175; Grant sponsor: NCI R21 and Training Grants; Grant numbers: 1R21CA139386, T32-CA009531.

*Correspondence to: Dr. Q. Ping Dou, The Prevention Program, Department of Pathology, Barbara Ann Karmanos Cancer Institute, School of Medicine, Wayne State University, 540.1 HWCRC, 4100 John R Road, Detroit, MI 48201. E-mail: doup@karmanos.org

Received 10 September 2009; Accepted 28 September 2009 • DOI 10.1002/jcb.22394 • © 2009 Wiley-Liss, Inc.
Published online 12 November 2009 in Wiley InterScience (www.interscience.wiley.com).

Owing to their anti-inflammatory and immunosuppressive properties, several gold(I) compounds, such as auranofin, have been employed in the treatment of rheumatoid arthritis and investigated as anti-tumor agents [Ward, 1988]. Auranofin, a gold(I)–thioglucose derivative, and a number of its analogs were evaluated for their in vitro cytotoxic activities, showing to be active against a panel of tumor cells, but not against solid tumors [Kostova, 2006]. Following these encouraging results, a series of new gold compounds were synthesized and some of them, such as digold(I)–phosphino complexes, exhibited improved anti-tumor activity [Sadler and Sue, 1994].

Since gold(III) is isoelectronic as platinum(II) (i.e., d^8 electronic configuration) and tetracoordinate gold(III) complexes exhibit the same square-planar geometry as platinum (II) complexes [Ronconi et al., 2006], the anti-cancer activity of gold(III) complexes has been investigated. Recently, a number of gold(III)–dithiocarbamato complexes were reported to be one- to fourfold more cytotoxic than cisplatin and were able to overcome resistance to cisplatin itself [Ronconi et al., 2005]. Further studies on interactions of these gold(III) complexes with both isolated DNA, the primary cellular target of platinum(II) complexes, and RNA suggest a distinct mechanism of action [Ronconi et al., 2006]. Thus, this observation has prompted us to search for alternative cellular targets, such as proteins, to gain insight into the biological effects of gold compounds.

The ubiquitin–proteasome pathway is a tightly controlled process responsible for degradation of intracellular proteins involved in various cellular processes, including cell-cycle regulation and apoptosis [Orlowski and Dees, 2003]. Because of its broad involvement in fundamental biochemical processes, the ubiquitin–proteasome pathway is associated with the pathological states of several human diseases, including cancer [Spataro et al., 1998]. The 20S proteasome serves as a multicatalytic protease of the 26S proteasome and contains at least three critical enzymatic activities: chymotrypsin-like, trypsin-like, and caspase-like [Seemuller et al., 1995]. Since inhibition of proteasomal chymotrypsin-like activity is associated with apoptosis induction in tumor cells, the ubiquitin–proteasome pathway has become an attractive target for the development of novel anti-cancer drugs [An et al., 1998; Almond and Cohen, 2002; Adams, 2003]. Validation of such a target for cancer therapy came with the approval of bortezomib for the treatment of multiple myeloma [Dou and Goldfarb, 2002].

Reactive oxygen species (ROS) are known to play a dual role in biological systems, by producing deleterious or beneficial effects in living organisms [Valko et al., 2006]. Despite the presence of an effective antioxidant system to counter the adverse effects of ROS, induction of oxidative damage to cellular proteins plays an important role in the pathogenesis of various diseases, including cancer [Halliwell, 1996; Poli et al., 2004; Valko et al., 2006]. The proteasome itself has been shown to be susceptible to oxidative modification and inactivation [Szweda et al., 2002]. Recent studies have shown that a number of anti-cancer agents, including proteasome inhibitors could induce cell death through an increase in oxidative stress [Adachi et al., 2002; Perez-Galan et al., 2006]. However, the mechanism by which this takes place has yet to be delineated. It was suggested that neoplastic cells, with a heightened rate of endogenous ROS activity may be more susceptible to anti-cancer drugs, by either increasing ROS generation or decreasing its ROS-scavenging capability [Schumaker, 2006]. Accordingly, induction of ROS toxicity in tumor cells may serve as effective means in distinguishing normal from malignant cells and provide a viable therapeutic strategy.

Recently, we have demonstrated that dithiocarbamato derivatives of several transition metals, including Au(III), Zn(II), and Cu(II), act as potent proteasome inhibitors in tumor cells [Daniel et al., 2005; Milacic et al., 2006, 2008a]. Proteasome inhibition induced by these complexes was associated with apoptosis induction in vitro and in vivo. In the current study, we attempt to gain further insight into the biological activity of gold–dithiocarbamato complexes in which the metal centers exhibit different oxidation states. We found that both AUL12 (with gold in oxidation state 3^+) and its gold(I) counterpart $[Au(ESDT)]_2$ (AUL15) are able to inhibit the chymotrypsin-like activity of a purified 20S proteasome ($IC_{50} = 1.13$ and 17.72 μmol/L, respectively) and 26S proteasome in intact human breast cancer MDA-MB-231 cells, but with significantly different potencies. Inhibition of the proteasome activity, as demonstrated by higher levels of ubiquitinated proteins and the proteasome target IκB-α, and cell death was induced to a greater extent by AUL12, compared to AUL15. Addition of a reducing agent significantly blocked proteasome inhibition and cell death induced by both investigated gold complexes. Furthermore, treatment with AUL12 was associated with the production of ROS in breast cancer cells, whereas the treatment with gold(I) analog AUL15 had little effect.

MATERIALS AND METHODS

MATERIALS

Gold–dithiocarbamato complexes AUL12 and AUL15 were synthesized and characterized as previously described [Ronconi et al., 2005]. 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dithiothreitol (DTT), *N*-acetyl-L-cysteine (NAC), DMSO, and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). DMEM/F-12, penicillin, streptomycin, and ROS detection kit were purchased from Invitrogen (Carlsbad, CA). Purified rabbit 20S proteasome was purchased from Boston Biochem (Cambridge, MA). Flurogenic peptide substrate, Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) was purchased 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, IκB-α, ubiquitin, goat polyclonal antibody against actin, and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

CELL CULTURE AND WHOLE-CELL EXTRACT PREPARATION

Human breast cancer MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/F-12 supplemented with 10% fetal bovine serum and 100 U/ml of penicillin and 100 μg/ml of streptomycin. All cells were grown at 37°C in a humidified incubator with a 5% CO_2-enriched atmosphere. A whole-cell extract was prepared as previously described [Daniel et al., 2005].

INHIBITION OF THE PURIFIED 20S PROTEASOMAL ACTIVITY BY GOLD COMPOUNDS

Purified rabbit 20S proteasome (35 ng) was incubated with 20 μmol/L of the fluorogenic substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) in 100 μl assay buffer (25 mmol/L Tris–HCl, pH 7.5) for 2 h at 37°C in the presence of either gold compound at different concentrations or equivalent (v/v) percentage of DMSO as control. After incubation, production of hydrolyzed AMC groups was measured with a Wallac Victor^3 multilabel counter with an excitation filter of 365 nm and emission filter of 460 nm.

PROTEASOME ACTIVITY ASSAY IN INTACT HUMAN BREAST CANCER MDA-MB-231 CELLS

Human breast cancer MDA-MB-231 cells were grown to 70–80% confluency, treated with indicated compound or DMSO as a control 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

MDA-MB-231 cells were seeded in triplicate in a 96-well plate and grown until 70–80% confluence, followed by treatment with each compound or DMSO (as a control) for 24 h. After that, the MTT assay was done as previously described [Daniel et al., 2005].

WESTERN BLOT ANALYSIS

MDA-MB-231 breast cancer cells were treated, harvested, and lysed. Cell lysates (40–50 μg) were separated by SDS–PAGE and transferred to a nitrocellulose membrane followed by visualization using the HyGLO chemiluminescent HRP detection reagent from Denville Scientific (Metuchin, NJ), as previously described [Chen et al., 2005].

CELLULAR MORPHOLOGICAL AND ROS DETECTION ANALYSIS

A Zeiss Axiovert 25 microscope was used for all microscopic imaging with either phase-contrast for cellular morphology or fluorescence for ROS detection. MDA-MB-231 cells were seeded in a six-well plate on top of a cover slip and grown to 70–80% confluency, followed by treatment with either AUL12 or AUL15, or DMSO for 3.5 h. *t*-Butylhydroperoxide (TBHP), a common ROS inducer, at 100 μM was used as a positive control. Cells that adhered to the cover slips were gently washed once with warm HBSS/Ca/Mg followed by labeling with 1 ml of 25 μmol/L carboxy-H_2DCFDA and 25 min incubation at 37°C. Then, 1.0 μmol/L of Hoechst 33342 was added to the cells and incubated for another 5 min in the dark. The cover slips were gently washed three times with warm HBSS/Ca/Mg before ROS generation was examined using a Zeiss confocal laser microscope.

ROS DETECTION BY FACS ANALYSIS

MDA-MB-231 cells were plated in p100 dishes and grown to 70–80% confluency, followed by treatment with AUL12 or AUL15, or DMSO control at the indicated concentrations for 3.5 h. Cells were then washed twice with warm HBSS/Ca/Mg followed by incubation with 25 μmol/L of carboxy-H_2DCFDA in a working solution of HBSS/Ca/Mg for 30 min at 37°C protected from light. Cells were then washed with warm HBSS/Ca/Mg and harvested. Prepared samples were then used to measure for ROS induction by FACS analysis.

ANNEXIN V-FITC BINDING ASSAY

The Annexin V-FITC binding assay was performed using the FITC Annexin V Apoptosis detection kit from BD Biosciences (San Jose, CA). MDA-MB-231 cells were treated with AUL12 or AUL15 at 10–30 μmol/L or DMSO as a control for 4 h. Harvested cells were washed with cold PBS and resuspended with 1× binding buffer, followed by Annexin V-FITC incubation for 15 min and PI staining for another 15 min at 4°C in the dark. The apoptosis indices were detected by flow cytometry.

RESULTS

GOLD(I)–DITHIOCARBAMATO COMPLEX AUL15 EXHIBITS DECREASED POTENCY IN INHIBITING PROLIFERATION OF HUMAN BREAST CANCER CELLS COMPARED TO ITS GOLD(III) ANALOG AUL12

We have previously shown that the gold(III)–dithiocarbamato complex [$AuBr_2$(DMDT)] exhibits potent anti-proliferative activity against different breast cancer cell lines and anti-tumor activity in nude mice bearing breast cancer xenografts [Milacic et al., 2006]. Moreover, our results showed that the cellular proteasome is one of the major targets and that proteasomal inhibition contributes to this gold(III)-mediated cell death. Based on these positive data, we set out to investigate two other gold–dithiocarbamato derivatives, namely AUL12 and AUL15 (Fig. 1A), whose anti-cancer activity was previously reported [Ronconi et al., 2005, 2006], in order to elucidate the importance of the oxidation state of the gold center (3^+ and 1^+, respectively). More importantly, we investigated a potential mechanism of action that may shed insight into the biological effects mediated by gold(III)- and gold(I)-containing compounds.

We first tested the growth-inhibitory effect of both gold compounds, with the solvent DMSO as a control, toward the highly aggressive breast cancer MDA-MB-231 cell line treated for 24 h, followed by an MTT assay. Although both compounds inhibited cell proliferation in a dose-dependent manner, AUL15 was less potent than AUL12 (Fig. 1B). For example, when cells were treated with 10 μmol/L of AUL15 or AUL12, cell proliferation was inhibited by 35% and 70%, respectively (Fig. 1B). The IC_{50} values of AUL15 and AUL12 were calculated to be 13.5 and 4.5 μmol/L, respectively (Fig. 1B).

GOLD(I)–DITHIOCARBAMAOTO COMPLEX AUL15 EXHIBITS LOWER PROTEASOME-INHIBITORY ACTIVITY THAN ITS GOLD(III) ANALOG AUL12

We have previously shown that the gold(III) compound [$AuBr_2$(DMDT)] could inhibit the chymotrypsin-like activity of purified proteasome [Milacic et al., 2006]. Therefore, we hypothesized that AUL12 could be similarly capable of targeting and inhibiting the proteasome in vitro. For comparison purposes, the gold (I) counterpart AUL15 was tested under the same experimental conditions. To provide direct evidence for this, we incubated a purified rabbit 20S proteasome with either AUL12 or AUL15 at various concentrations, with DMSO as a control, followed by

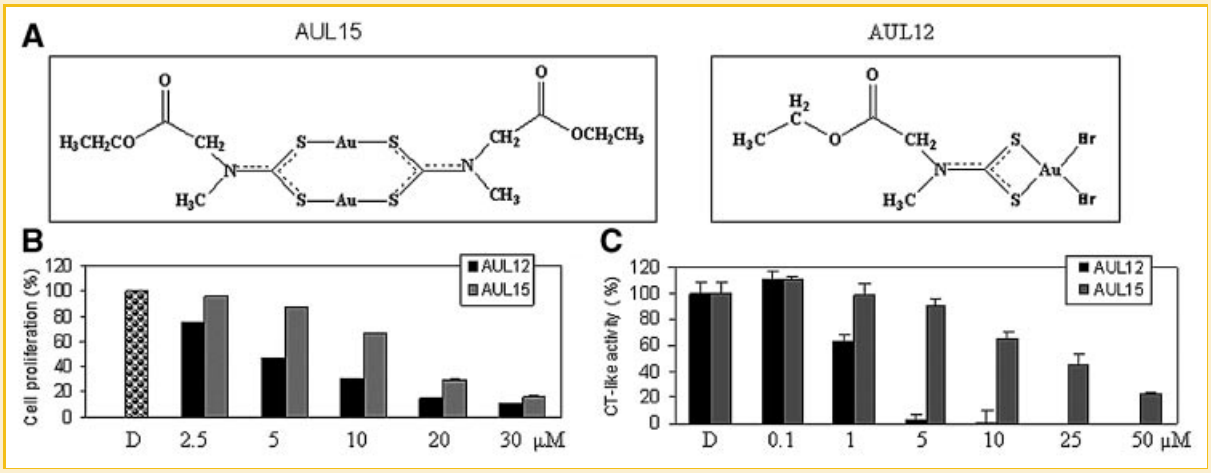

Fig. 1. The investigated gold compounds, AUL12 and AUL15, inhibit the proliferation of MDA-MB-231 breast cancer cells and the activity of purified 20S proteasome. A: Chemical structures of $[AuBr_2(ESDT)]$ (AUL12) and $[Au(ESDT)]_2$ (AUL15). B: Anti-proliferative effects of the investigated gold compounds. MDA-MB-231 cells were treated for 24 h with either AUL12 or AUL15 at indicated concentrations, with DMSO as a control. After 24 h, the medium was removed and cells were treated with the MTT reagent. Columns are average of three triplicate experiments. C: Inhibition of the chymotrypsin-like activity of purified 20S proteasome in the presence of AUL12 and AUL15 at the indicated concentrations, with DMSO as a control, measured as described in the Materials and Methods Section.

measurement of proteasomal activity. We found that AUL15 inhibited the proteasomal chymotrypsin-like activity, but with decreased potency compared to AUL12, with IC_{50} values of 17.7 and 1.13 μmol/L, respectively (Fig. 1C).

To investigate whether AUL12 and AUL15 could inhibit proteasomal activity in intact cells, breast cancer MDA-MB-231 cells were treated with 10–30 μmol/L of each compound for 4 and 24 h. Cells were treated with DMSO as control. After the treatment, the cell extracts were used to measure proteasomal chymotrypsin-like activity and accumulation of ubiquitinated proteins and proteasome target protein IκB-α. We found that AUL15 had no proteasome-inhibitory effect after 4 h of treatment, even at the highest concentration tested (Fig. 2A). However, after 24 h, it caused proteasome inhibition by 30% and 70% at 20 and 30 μmol/L, respectively (Fig. 2B). Consistent with this decreased level of proteasomal chymotrypsin-like activity by AUL15 at 24 h, accumulation of ubiquitinated IκB-α was found (Fig. 3B). Interestingly, accumulation of ubiquitinated proteins induced by AUL15 was detected after both 4 and 24 h (Fig. 3A). Since accumulation of ubiquitinated proteins is a transient process, the dose-dependent effect was not seen at 4 and 24 h, the two time points selected in this experiment. In comparison, cells treated with AUL12 at both 20 and 30 μmol/L significantly inhibited proteasomal chymotrypsin-like activity at both early (4 h) and late time points (24 h) (Fig. 2A,B). In addition, accumulation of ubiquitinated proteins and higher levels of IκB-α and its ubiqutinated form were apparent at both time points (Fig. 3A,B).

GOLD(I)–DITHIOCARBAMATO COMPLEX AUL15 EXHIBITS LOWER CELL DEATH-INDUCING ACTIVITY THAN ITS GOLD(III) ANALOG AUL12 IN INTACT BREAST CANCER MDA-MB-231 CELLS

To investigate whether inhibition of proteasomal chymotrypsin-like activity was associated with apoptosis or cell death induction, morphological changes, PARP cleavage, Annexin V-FITC, and TUNEL were examined. Changes in cell morphology (shrunken, rounded up, and characteristic apoptotic blebbing) were apparent mostly during later time points of the treatment with increasing concentrations of AUL15 (Fig. 2D vs. C). Furthermore, we noticed that cells treated with 10 μmol/L AUL12 started rounding up even after 4 h treatment and the effect was greatly enhanced after the treatment with higher concentrations (Fig. 2C). These cell-death-associated morphological changes were highly magnified after 24 h; rendering cells predominately rounded up and detached (Fig. 2D).

To study whether the observed cell death is related to apoptosis, we performed PARP cleavage assay. Treatment of cells with AUL15 at 10–30 μM for 4 h induced the cleavage of the intact PARP protein (p116 kDa) into a characteristic p65 fragment (Fig. 3C), a by-product of calpain cleavage [Pink et al., 2000]. The complete PARP cleavage was observed when AUL15 was used at higher concentrations for 24 h (Fig. 3C). We found the cleavage of the intact PARP protein (p116 kDa) into a characteristic p65 fragment in cells treated with either AUL 15 or AUL 12 in both time- and dose-dependent manners (Fig. 3C). It is notable to point out that treatment of cells with gold(I) complex AUL15 resulted in PARP disappearance mainly when higher concentrations were used (Fig. 3C). On the other hand, treatment with the gold(III) counterpart AUL12, at both early and late time points and at increasing concentrations resulted in complete disappearance of full-length PARP and higher levels of the cleaved p65 fragment (Fig. 3C).

We also performed FITC Annexin V staining assay. Treatment with AUL15 at 10 μM for 4 h induced ~21% of cells undergoing late apoptosis while 6% in non-apoptotic death (Fig. 2E). When AUL15 was increased to 20 μM, late apoptotic cells and non-apoptotic cell death were increased to 31% and 27%, respectively (Fig. 2E). However, further increase of AUL15 to 30 μM induced 72% non-apoptotic cell death (Fig. 2E). Treatment of cells with AUL12 at 10 μM for 4 h induced 40% late apoptotic cell death and 25% non-apoptotic cell death, more potent than AUL15 at the same conditions (21% and 6%, respectively; Fig. 2E). Similar to AUL15, when AUL12 was used at increased concentrations, the apoptotic death population was

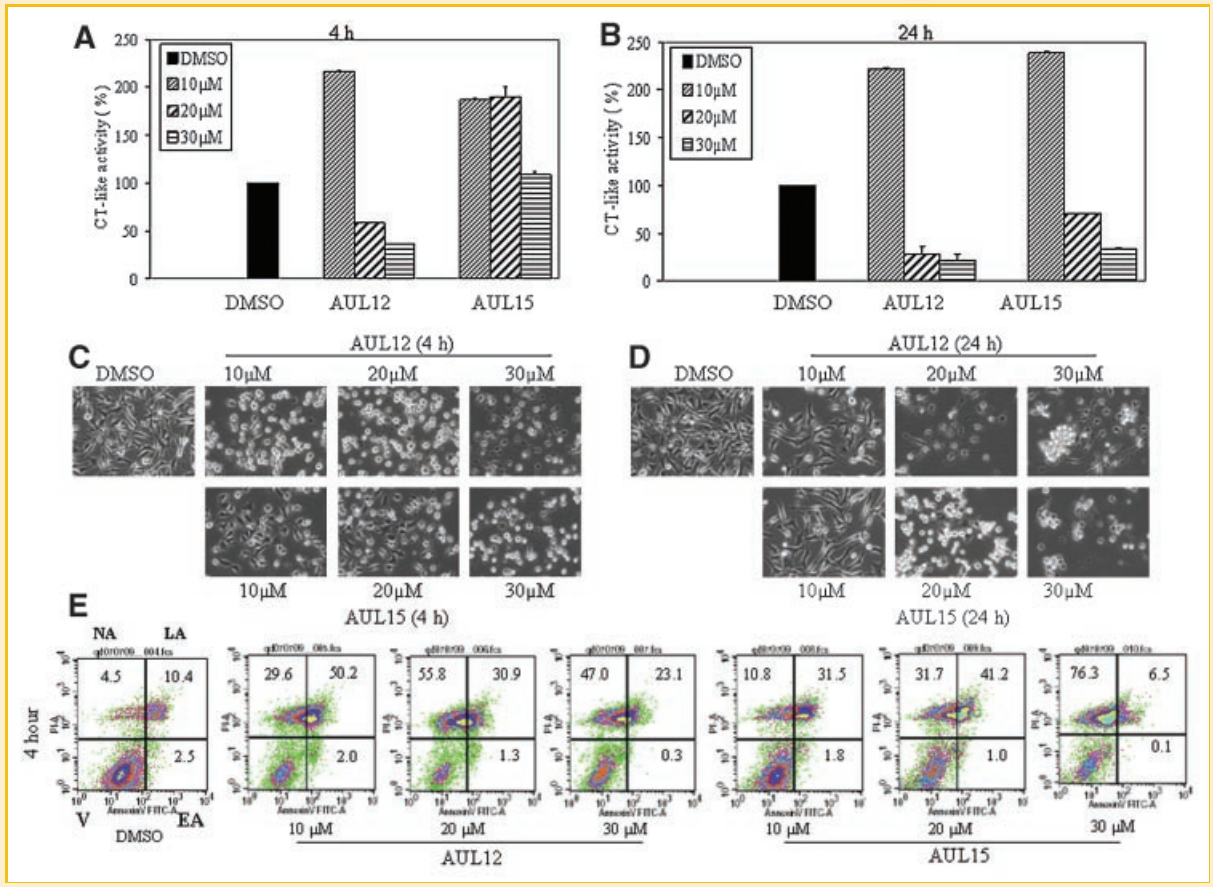

Fig. 2. Inhibition of proteasomal chymotrypsin-like activity and induction of cell death by gold compounds in MDA-MB-231 cells. Breast cancer MDA-MB-231 cells were treated with indicated concentrations of AUL12 or AUL15 for 4 h (A,C,E) or 24 h (B,D), with DMSO as a control, followed by measurement of the proteasomal chymotrypsin-like activity (A,B) and visualization of morphological changes (C,D), as described in the Materials and Methods Section. E: Annexin V-FITC binding assay was performed to quantify the number of apoptotic cells. The lower right (Annexin V-FITC+/PI−) was considered early stage of apoptotic cells (EA) and upright part (Annexin V-FITC+/PI+) was considered as late stage of apoptotic cells (LA). The lower left part (Annexin V-FITC−/PI−) was considered as viable cells (V) and the upper left part (Annexin V-FITC−/PI+) was considered non-apoptotic cell death (NA).

decreased while the non-apoptotic death cells were increased (Fig. 2E). We also investigated whether treatment with gold compound-induced cytotoxicity is related to DNA damage. Treatment with AUL15 for 4 h at only 30 μM induced TUNEL positivity by ~10% while AUL12 at only 20 and 30 μM generated 2.0% and 30% TUNEL-positive cells (data not shown). Taken together, our data suggest that these gold–dithiocarbamato complexes induced various types of cell death, depending on the status of gold compound, concentrations, and treatment time. However, these data suggest that gold(III) compound has higher proteasome-inhibitory activity and induced higher level of cell death compared to gold(I)–dithiocarbamate treatment (see the Discussion Section).

We and others have previously shown that associated with the cell death commitment, Bax protein (p21/Bax) could be cleaved by calpain, producing a p18/Bax fragment, which then forms a homodimer p36/Bax [Gao and Dou, 2000; Wood and Newcomb, 2000]. Since treatment with gold compounds can lead to the calpain activation and p65/PARP fragment production (Fig. 3C), we would then expect the appearance of the Bax/p36 homodimer upon treatment. Under our experimental conditions, using breast cancer MDA-MB-231 cells (with DMSO as a control), we detected two forms of Bax protein: p21 and p36 (Fig. 3D). When the cells were treated with AUL15, only the treatment with high concentrations for 24 h resulted in higher levels of p18/Bax and p36/Bax (Fig. 3D). In contrast, treatment with AUL12 even at the lowest concentration resulted in higher levels of p18/Bax and p36/Bax during both early and late time points (Fig. 3D). This effect was more pronounced upon treatment with higher concentrations of AUL12 (Fig. 3D). Together, these results show that the gold(III) compound is able to inhibit proteasomal chymotrypsin-like activity, activate calpain, and induce cell death in human breast cancer MDA-MB-231 cells with a higher degree of activity compared to its gold(I) counterpart.

PROTEASOME INHIBITION AND CELL DEATH INDUCTION BY GOLD(I)– AND GOLD(III)–DITHIOCARBAMATO DERIVATIVES IS BLOCKED BY THE REDUCING AGENTS DTT AND NAC

It has been shown that some metals can oxidize cellular proteins and that this process could be blocked by reducing agents such as DTT and NAC [Godfrey et al., 1994; Rattan and Arad, 1998]. Since both AUL12 and AUL15 could inhibit the chymotrypsin-like activity of

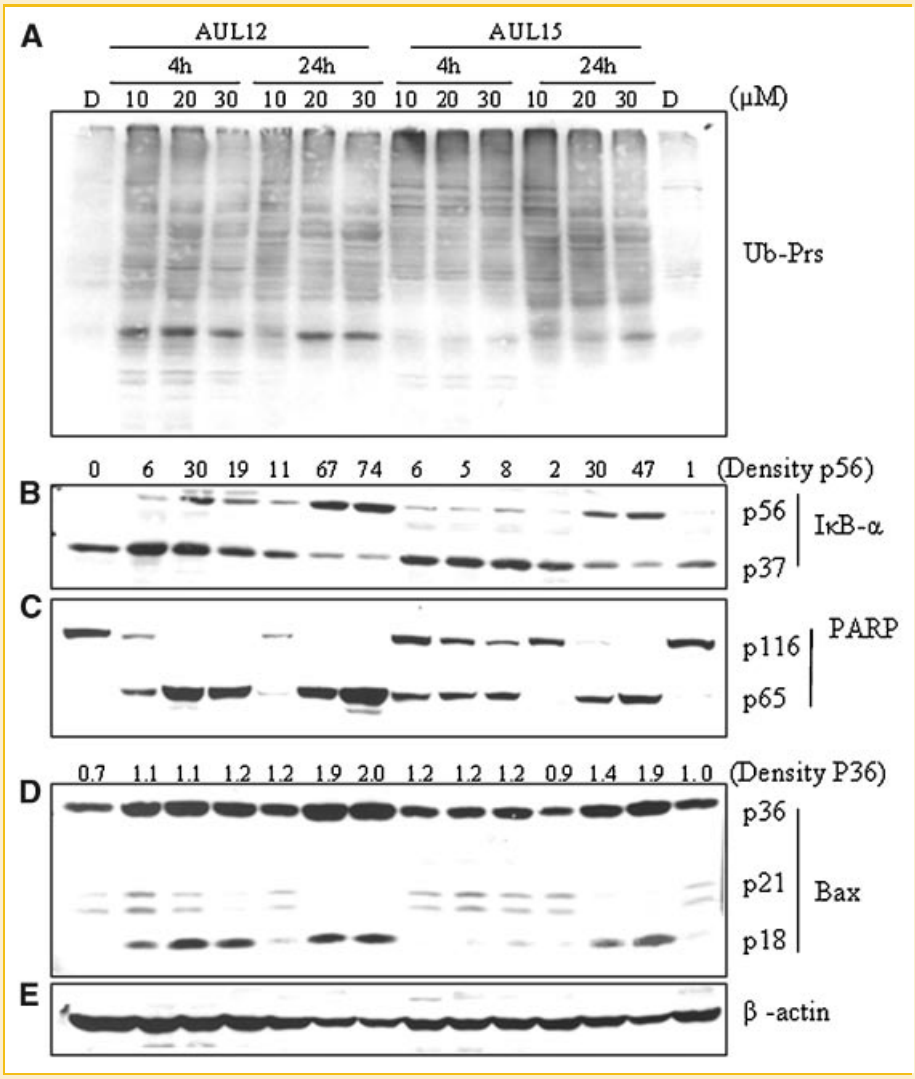

Fig. 3. Western blot analysis. Breast cancer MDA-MB-231 cells were treated with indicated concentrations of AUL12 or AUL15 for 4 or 24 h, followed by performance of Western blot analysis. DMSO solvent was used as a control. A: Accumulation of ubiquinated proteins; (B) accumulation of IκB-α and ubiquitinated form of IκB-α; (C) PARP cleavage; (D) accumulation of Bax isoforms; and (E) actin used as a loading control.

purified rabbit 20S proteasome (Fig. 1C) and cellular 26S proteasome of cultured breast cancer cells (Figs. 2 and 3), we then tested whether reducing agents such as DTT and NAC could affect these events. We found that inhibition of purified 20S proteasome activity by AUL15 was reversed in a dose-dependent manner by addition of DTT and was essentially restored to basal levels with 300 μmol/L DTT (Fig. 4A). Similarly, inhibition of purified 20S proteasome by AUL12 could also be reversed by ~80% upon co-incubation of 50 μmol/L of DTT (Fig. 4B).

The effect of NAC on AUL12 and AUL15-induced proteasome inhibition in intact MDA-MB-231 cells was also investigated. We found that NAC at 150 μmol/L, effectively restored proteasome activity in the cells treated with 30 μmol/L AUL15 (Fig. 5A). Additionally, 100 μmol/L NAC could block proteasome inhibition induced by 20 μmol/L AUL12 after 24 h (Fig. 5A). Consistently, co-treatment with each gold compound and NAC blocked accumulation of ubiquitinated proteins and the ubiquitinated form of IκB-α (Fig. 5B). Importantly, NAC almost completely blocked gold(I)- and gold(III)-induced production of p65 PARP cleavage (Fig. 5B) and cellular morphological changes (Fig. 5C). Furthermore, addition of NAC also inhibited the increased levels of p18/Bax to p36/Bax induced by each gold compound, suggesting that NAC inhibits AUL12 and AUL15-induced calpain activation (Fig. 5B).

PROTEASOME INHIBITION AND APOPTOSIS INDUCTION BY GOLD(III) BUT NOT GOLD(I)–DITHIOCARBAMATE IS MEDIATED THROUGH ACTIVATION OF REACTIVE OXYGEN SPECIES (ROS)

It has been demonstrated that some chemotherapeutic agents, including proteasome inhibitors, could mediate cell death through increase in oxidative stress [Perez-Galan et al., 2006]. Our data show that gold(I)– and gold(III)–dithiocarbamato derivatives can inhibit the proteasomal chymotrypsin-like activity and induce cell death, which can be completely reversed by the addition of a reducing agent. We next set out to determine if the induction of ROS is

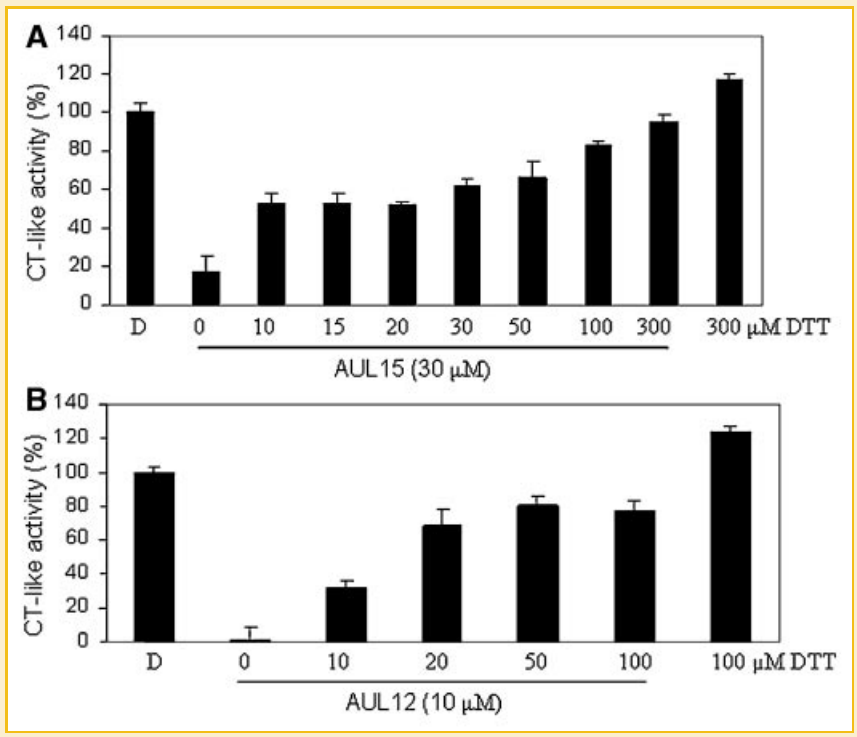

Fig. 4. Inhibition of purified 20S proteasomal activity by gold compounds is blocked by DTT. Purified rabbit 20S proteasome was incubated with a peptide substrate for the proteasomal chymotrypsin-like activity and indicated concentrations of AUL15 (A) or AUL12 (B) in the absence or presence of DTT at indicated concentrations for 2 h, followed by the measurement of production of hydrolyzed AMC groups (see the Materials and Methods Section for details). DMSO was used as the solvent control.

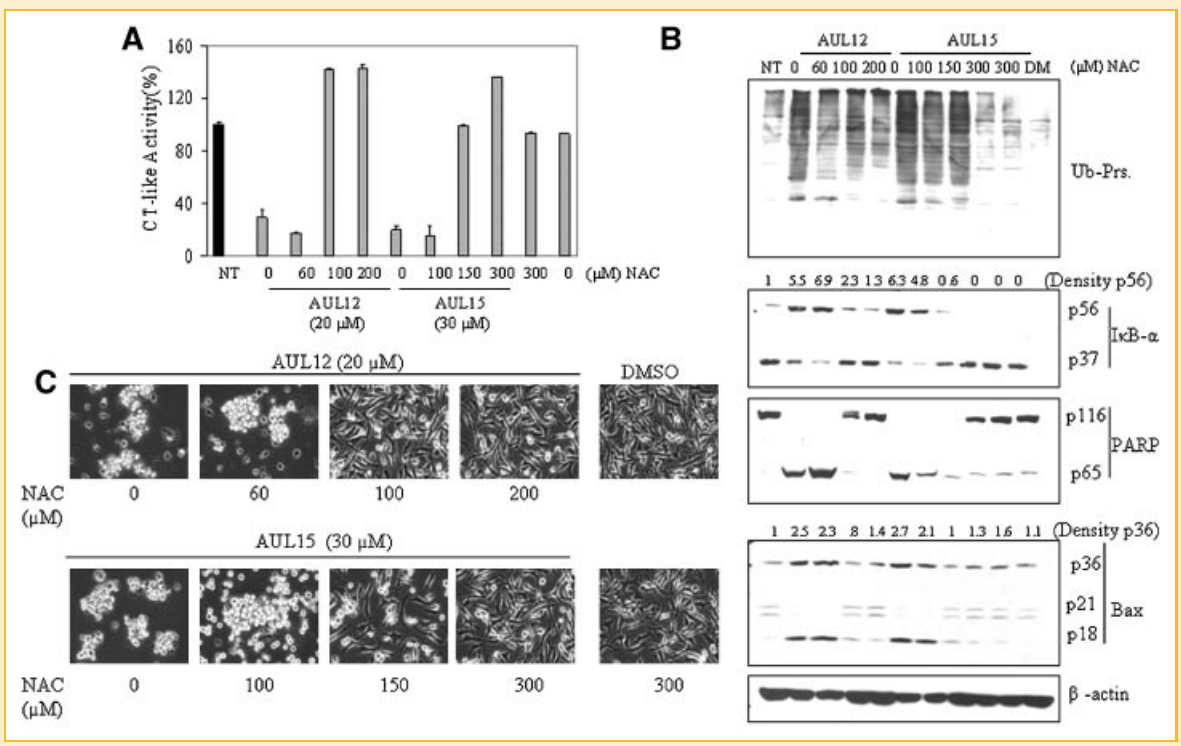

Fig. 5. Proteasome inhibition and apoptosis induction by AUL12 and AUL15 is blocked by NAC in intact MDA-MB-231 cells. Breast cancer MDA-MB-231 cells were treated with indicated concentrations of AUL12 or AUL15 for 24 h, in the absence or presence of various concentrations of NAC, followed by measurement of the proteasomal chymotrypsin (CT)-like activity (A), Western blot analysis (B), and apoptotic morphological changes (C) as visualized by phase-contrast imaging (100× magnification).

responsible for cell death mediated by these complexes. Breast cancer MDA-MB-231 cells were treated with both 20 and 30 μmol/L of AUL12 or AUL15 for 3.5 h along with the oxidation-sensitive probe carboxy-H_2DCFDA, followed by analysis of ROS induction by fluorescence microscopy and FACS analysis. DMSO was used as the negative control and TBHP as positive control under the same experimental conditions. Our results show that treatment with AUL15, at both concentrations, was unable to produce reasonably detectable levels of green fluorescence (1.0–1.3% ROS-positive cells; Fig. 6A,B). However, treatment with AUL12 at both

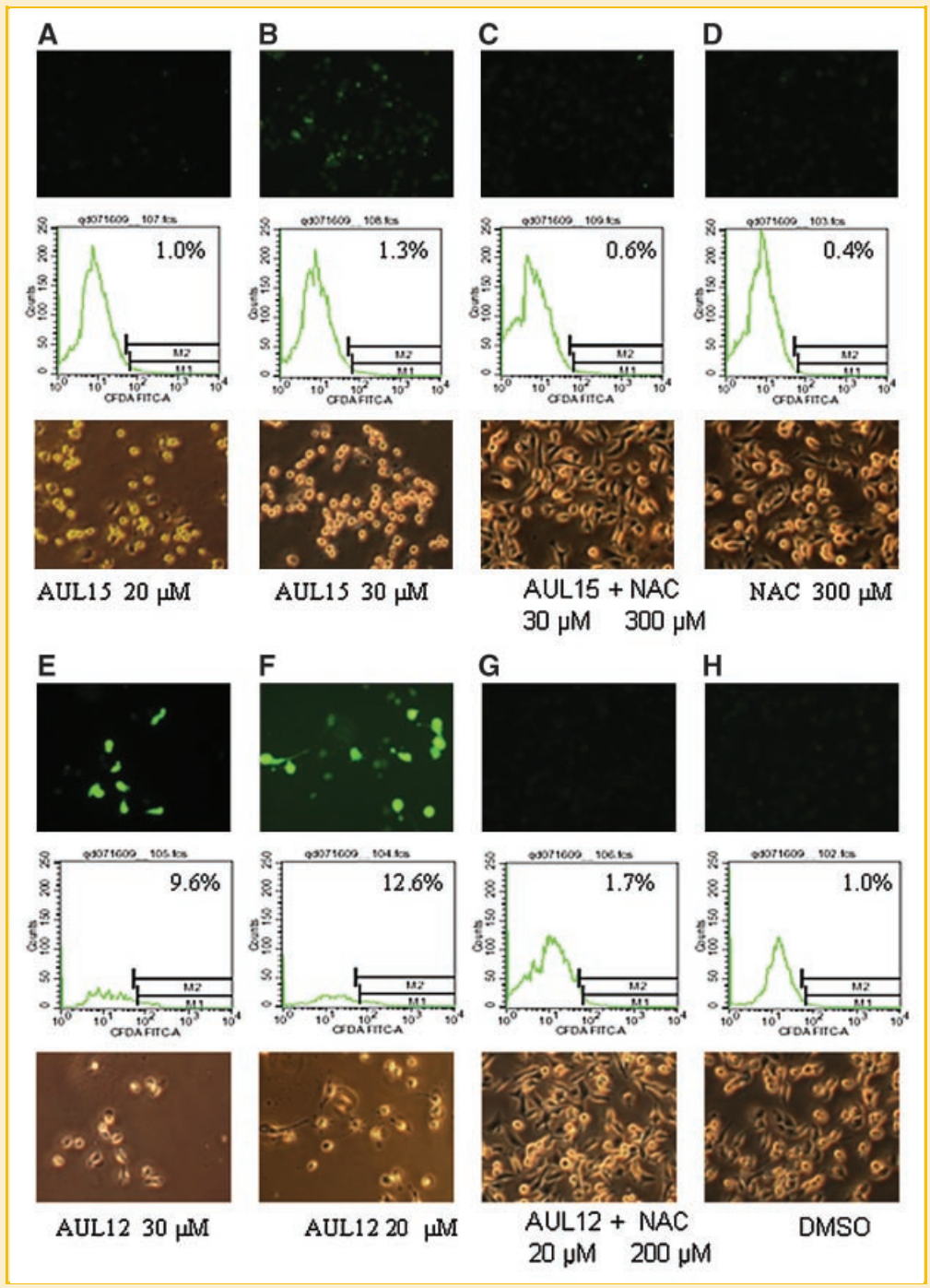

Fig. 6. AUL12 [Au(III)] but not AUL15 [Au(I)] induces ROS production in breast cancer cells and is effectively blocked by the addition of NAC. ROS formation in MDA-MB-231 cells was determined using the oxidation-sensitive probe (carboxy-H_2DCFDA). Cells were treated with AUL12 or AUL15 at the indicated concentrations for 3.5 h with or without the addition of NAC. DMSO and NAC alone were used as negative controls under the same experimental conditions. ROS generation was examined using a Zeiss confocal laser microscope (first rows) and FACS analysis (second rows) as explained in the Materials and Methods Section. The morphological changes of the same cells were also shown (third rows) by phase contrast imaging (100x magnification).

concentrations resulted in the enhancement of bright green fluorescence indicative of the production of ROS (9.6–12.6% ROS-positive cells; Fig. 6E,F).

Since we have shown that the addition of a reducing agent could completely block the effect of proteasome inhibition and cell death induction, we next investigated whether the addition of NAC could block the production of ROS induced by gold(III)–dithiocarbamate. Our data show that production of ROS in cells treated with the gold(III) complex was almost completely blocked by the addition of 200 μmol/L NAC (1.7% ROS-positive cells; Fig. 6G). Moreover, the treatment of cells with the gold(I) analog, with or without co-treatment of NAC failed to induce any significant levels of ROS, thus confirming that the gold(I) derivative is redox inactive (Fig. 6A–C). These results clearly suggest that induction of oxidative stress by gold(III), but not gold(I), is at least partially responsible for its biological activity which can be effectively inhibited through the addition of a reducing agent.

DISCUSSION

The clinical impact of platinum-based complexes has precipitated the investigation of other metals which broadens the spectrum of activity and presents modes of action that differ from cisplatin. Although the mechanism of cisplatin has been readily understood, the mechanism of gold compounds responsible for their anti-tumor activity is still under debate. A number of studies suggested that proteins, rather than DNA, may be a more suitable target for gold-containing complexes. Some of the suggested targets for gold compounds include S-donor ligands such as glutathione (GSH) and cysteine [Milacic et al., 2008b], bovine serum albumin [He and Carter, 1992], thioredoxin reductase [Engman et al., 2006], and ERK pathway [Saggioro et al., 2007]. Based on these studies, it was proposed that selective modification of surface protein residues by gold(III) complexes is more likely to be responsible for their biological activity.

We have previously shown that the gold(III)–dithiocarbamato derivative ([$AuBr_2$(DMDT)]) was able to inhibit the proteasomal activity and induce apoptosis in cultured breast cancer cells and mice bearing breast cancer xenografts [Milacic et al., 2006]. Furthermore, proteasomal inhibition and apoptosis induction could be effectively reversed by the addition of a reducing agent [Milacic et al., 2006]. The reversal of [$AuBr_2$(DMDT)]-mediated proteasomal inhibition by a reducing agent could be a result of the reaction that pulls the complex away from the proteasome, thereby preventing it from binding and inhibiting the proteasome. This possibility was supported by previous findings that gold(III) complexes can bind S-donor ligands, such as GSH and cysteine, and cleave their disulfide bond(s) [Zou et al., 1999]. It is also possible that NAC or DTT could reduce gold(III) to gold(I), an oxidation state that does not have or has lower affinity for binding and inhibiting the proteasome. Another suggested possibility was based on the report that gold(III) porphyrin 1a induces intracellular oxidation, altering reduced GSH levels in the cell [Wang et al., 2005]. Therefore, a possibility that [$AuBr_2$(DMDT)] might stimulate production of ROS that oxidize and inactivate the proteasome was proposed [Milacic et al., 2006]. This hypothesis was supported by a well-established susceptibility of the proteasome to oxidative modification and inactivation upon exposure to free radical-generating systems [Szweda et al., 2002].

In order to evaluate the importance of the 3^+ oxidation state for the mechanism of action and overall anti-tumor activity of gold compounds, we compared two gold–dithiocarbamato derivatives with different oxidation states of the gold centers, to provide insights into their possible mechanism of action.

When comparing their anti-proliferative activities, we found that AuL15 was less potent than AUL12 against breast cancer MDA-MB-231 cells ($IC_{50} = 13.5$ and 4.5 μmol/L, respectively; Fig. 1B). Since both gold compounds own the same ESDT ligand, this finding suggests that the observed effect could be mediated by the different oxidation states of the gold centers.

We then set out to investigate if they share the same molecular target as [$AuBr_2$(DMDT)—the proteasome]. We performed a cell-free activity assay using a purified 20S proteasome incubated with each gold compound (Fig. 1C). Interestingly, we found that both complexes inhibited the proteasomal chymotrypsin-like activity, but at significantly different potencies. Similar to their anti-proliferative activities, AUL15 was less potent in inhibiting the proteasome compared to AUL12 ($IC_{50} = 17.7$ and 1.13 μmol/L, respectively; Fig. 1C).

To investigate their capability to target and inhibit the cellular proteasome, we treated intact MDA-MB-231 cells and found a similar different effect in activity. Proteasomal inhibition was measured by decreased proteasomal activity, increased levels of ubiquitinated proteins, and accumulation of ubiquitinated form of the proteasomal target protein IκB-α (Figs. 2A,B and 3A,B). Additionally, cell death induction associated with proteasomal inhibition was measured. We found that the effects of the gold(I) compound were again much less pronounced compared to its gold(III) counterpart in the context of cell morphological change, the cleaved PARP fragment p65, Annexin V-FITC staining, and TUNEL (Figs. 2 and 3 and data not shown). We found that at 4 h, cells round up and detachment was observed associated with both apoptotic and non-apoptotic death and calpain-mediated PARP cleavage in to fragment p65, but in the absence of TUNEL-positivity cells detected. This suggests that the cell death observed at 4 h is not apoptosis and it may be calpain-dependent but DNA-damage-independent necrosis. However, at 24 h, both apoptosis and such necrosis occurred. Therefore, both necrosis (or DNA-damage-independent cell death) and apoptosis could be induced by these gold compounds and gold(III) has a higher effect while AUL15 at higher concentrations induced more non-apoptotic cell death. Taken together, these findings suggest that the lower potency of AUL15 in inhibiting the proteasome is, at least partly, responsible for its decreased anti-proliferative and cell-death-inducing effects, compared to the gold(III) compound, AUL12. We have also found that effects of both gold compounds could be blocked by two different reducing agents (DTT and NAC): both were able to reverse the effects induced by the investigated gold complexes in a cell-free system and in intact MDA-MB-231 cells (Figs. 4 and 5). We noticed that the similar potent reversion by NAC on cells treated with AUL15 as compared with cells exposed to AUL12. We found that in addition to inhibition of ROS production, NAC could also react with both gold

compounds (unpublished data). Therefore, the reversion of gold compound-mediated effects by NAC could be due to two mechanisms: (i) ROS inhibition and (ii) direct binding of these two compounds.

It has been shown that many anti-cancer drugs exert their effect through oxidative stress, and that the proteasome is susceptible to oxidative modification and inactivation upon exposure to free radical generating systems [Szweda et al., 2002; Schumaker, 2006]. Therefore, in an attempt to explain the different potencies of AUL12 and AUL15, we set out to investigate whether they could induce the production of ROS. Our results show that treatment with gold(III) compound induces the production of ROS, whereas the gold(I) compound produces much less (Fig. 6E,F vs. A,B). Furthermore, ROS induction by AUL12 could be effectively blocked by the addition of the reducing agent NAC (Fig. 6G). These results show that ROS induced by gold(III) but not gold(I) compound could be, at least partially, responsible for the observed proteasome inhibition. The observation that the treatment with NAC and AUL12 could effectively block the production of ROS, argues that the treatment of NAC may increase the pool of ROS scavengers, thereby preserving cellular integrity. The observation that the gold(I) compound displayed a lower cytotoxic profile may point to the lower affinity of gold(I) to the proteasome, since this different effect was observed under cell-free conditions and intact cells. Similarly, it is conceivable that the gold(I) compound may react with all the populations of NAC or DTT molecules, which could partially explain the reversal of gold(I)-mediated events.

Our finding that both gold(I) and gold(III) complexes have the same molecular target is not surprising since it is well known that metal centers are essential for the biological activity of metal-containing proteins and enzymes, and that metals are often responsible for the activity of organic drugs. The classic example is cisplatin, which exerts its anti-tumor activity by interacting with DNA [Zhu et al., 2005] forming a unique lesion that has not been mimicked by any other organic drugs. However, it has also been known that the activity of metal complexes is not determined only by the presence of the metal, especially when metals exist in different oxidation states and have rich coordination chemistry [Sadler, 1982]. In that case, even subtle changes in the charge of a metal can result in a significant change in the geometry of the complex, leading to dramatic alterations of its biological properties. This might explain the significant differences in the effects of gold(I) and gold(III) compounds investigated here.

AUL12 and AUL15 show different coordination modes at the gold center, that is, tetracoordinate square-planar and dico-ordinate linear structure, respectively. It is worth noting that under physiological conditions, AUL12 undergoes hydrolysis, thus delivering two moles of halide per mole of starting complex. Moreover, it has been shown to undergo a subsequent reduction process within 24 h, leading to the corresponding dinuclear gold(I) analog AUL15 [Ronconi et al., 2006]. However, this reduction process should not affect its cytotoxic properties, since a cytotoxic effect was shown to be exerted mainly within the first 12 h at nanomolar concentrations [Ronconi et al., 2005] and within 4 h at micromolar concentrations. Altogether, these observations strongly suggest that different activities of gold(I) and gold(III) compounds investigated here is a result of the different oxidation states and co-ordination modes of the gold centers.

Overall, we hypothesize that there are at least two mechanisms responsible for the biological activity of these gold compounds, including ROS production and direct metal binding to the proteasome. The assertion that gold compounds and other metals can only act non-specifically and inhibit the proteasome by a secondary effect has been refuted by previous studies from our lab and others. It is notable to point out that treatment with gold(I) at 30 μM for 4 h resulted in the production of ~72% cells undergoing non-apoptotic-related cell death while only producing very low levels of ROS (Figs. 2E vs. 6B). We have also shown that zinc-containing dithiocarbamate derivatives could inhibit the proteasomal activity but could not be blocked by DTT or NAC, suggesting that these compounds are not acting through ROS (data not shown). Additionally, it has been published previously that zinc does not have a relevant oxidative strength [Amici et al., 2002]. Furthermore, we have also found that Sn could directly bind and inhibit the cellular proteasome [Shi et al., 2009]. Taken together, these results demonstrate that metal-containing compounds do not exert their effect non-specifically.

In conclusion, we found that both AUL12 and AUL15 inhibit the proteasome under cell-free conditions and in cultured breast cancer cells, but with different potencies. Furthermore, in both cases, proteasome inhibition and cell death induction can be effectively reversed by the addition of DTT or NAC. Interestingly, the gold(III) complex was able to induce production of ROS in intact breast cancer cells, which might be at least partly responsible for the proteasome-inhibitory effect. Although the gold(I) analog failed to stimulate ROS at any sustained levels, its proteasome-inhibitory and cell-death-inducing effects were also completely blocked by the reducing agent. However, further in-depth studies are required to delineate this phenomenon completely. Since up to date pharmacologically employed platinum-containing compounds are strongly associated with non-specific toxicity, the alternative of metal complexes, especially gold derivatives, as proteasome inhibitors, seems to be a promising approach in cancer therapy.

ACKNOWLEDGMENTS

This work was supported by Karmanos Cancer Institute of Wayne State University (to Q.P.D.), Department of Defense Breast Cancer Research Program Awards (W81XWH-04-1-0688 and DAMD17-03-1-0175 to Q.P.D.), NCI R21 and Training Grants (1R21CA139386 to Q.P.D. and T32-CA009531 to M.F.).

REFERENCES

Adachi M, Zhang Y, Zhao X, Minami T, Kawamura R, Hinoda Y, Imai K. 2002. Synergistic effect of histone deacetylase inhibitors FK228 and m-carboxycinnamic acid bis-hyrdoxamide with proteasome inhibitors PSI and PS-341 against gastrointestinal adenocarcinoma cells. Clin Cancer Res 10:3853–3862.

Adams J. 2003. Potential for proteasome inhibition in the treatment of cancer. Drug Discov Today 8:307–315.

Almond JB, Cohen GM. 2002. The proteasome: A novel target for cancer chemotherapy. Leukemia 16:433–443.

Amici M, Forti K, Nobili C, Lupidi G, Angeletti M, Fioretti E, Eleuteri AM. 2002. Effect of neurotoxic metal ions ion the proteolytic activities of the 20S proteasome from bovine brain. J Biol Inorg Chem 7:750–756.

An B, Goldfarb RH, Siman R, Dou QP. 1998. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ 5:1062–1075.

Chen D, Daniel KG, Chen MS, Kuhn DJ, Landis-Piwowar KR, Dou QP. 2005. Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. Biochem Pharmacol 69:1421–1432.

Criado JJ, Manzano JL, Rodriguez-Fernandex E. 2003. New organotropic compounds. Synthesis, characterization and reactivity of Pt(II) and Au(III) complexes with bile acids: DNA interactions and ''in vitro'' anticancer activity. J Inorg Biochem 96:311–320.

Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, Dou QP. 2005. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. Breast Cancer Res 7 (6): R897–R908.

Dou QP, Goldfarb RH. 2002. Bortezomib (Milenium Pharmaceuticals). IDrugs 8:828–834.

Engman L, McNaughton M, Gajewska M, Kumar S, Birmingham A, Powis G. 2006. Thioredoxin reductase and cancer cell growth inhibition by organogold(III) compounds. Anticancer Drugs 17:539–544.

Gao G, Dou QP. 2000. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. J Cell Biochem 80:53–72.

Godfrey EG, Stewart J, Dargie HJ, Reid JL, Dominiczak M, Hamilton CA, McMurray J. 1994. Effects of ACE inhibitors on oxidation of human low density lipoprotein. Br J Clin Pharmacol 37:63–66.

Halliwell B. 1996. Antioxidants in human health and disease. Annu Rev Nutr 16:33–50.

He XM, Carter DC. 1992. Atomic structure and chemistry of human serum albumin. Nature 358:209–215.

Kostova I. 2006. Gold coordination complexes as anticancer agents. Anticancer Agents Med Chem 6:19–32.

Milacic V, Chen D, Ronconi L, Landis-Piwowar KR, Fregona D, Dou QP. 2006. 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. Cancer Res 66:10478–10486.

Milacic V, Chen D, Giovagnini L, Diez A, Fregona D, Dou QP. 2008a. Pyrrolidine dithiocarbamate-zinc(II) and -copper(II) complexes induce apoptosis in tumor cells by inhibiting the proteasomal activity. Toxicol Appl Pharmacol 231:24–33.

Milacic V, Fregona D, Dou QP. 2008b. Gold complexes as prospective metal-based anticancer drugs. Histol Histopathol 23:101–108.

Orlowski RZ, Dees EC. 2003. The role of the ubiquitination-proteasome pathway in breast cancer: Applying drugs that affect the ubiquitin-proteasome pathway to the therapy of breast cancer. Breast Cancer Res 5:1–7.

Perez-Galan P, Roue G, Villamor N, Montserrat E, Campo E, Colomer D. 2006. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. Blood 107:257–264.

Pink JJ, Wuerzberger-Davis S, Tagliarino C, Planchon SM, Yang X, Froelich CJ, Boothman DA. 2000. Activation of a cysteine protease in MCF-7 and T47D breast cancer cells during beta-lapachone-mediated apoptosis. Exp Cell Res 255:144–155.

Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. 2004. Oxidative stress and cell signaling. Curr Med Chem 11:1163–1182.

Rattan AK, Arad Y. 1998. Temporal and kinetic determinants of the inhibition of LDL oxidation by N-acetylcysteine (NAC). Atherosclerosis 138:319–327.

Ronconi L, Giovagnini L, Marzano C, Bettio F, Graziani R, Pilloni G, Fregona D. 2005. Gold dithiocarbamate derivatives as potential antineoplastic agents: Design, spectroscopic properties, and in vitro antitumor activity. Inorg Chem 44:1867–1881.

Ronconi L, Marzano C, Zanello P, Corsini M, Miolo G, Macca C, Trevisan A, Fregona D. 2006. Gold(III) dithiocarbamate derivatives for the treatment of cancer; solution chemistry, DNA binding, and hemolytic properties. J Med Chem 49:1648–1657.

Sadler PJ. 1982. The comparative evaluation of the physical and chemical properties of gold compounds. J Rheumatol Suppl 8:71–80.

Sadler PJ, Sue RE. 1994. The chemistry of gold drugs. Met Based Drugs 1:107–144.

Saggioro D, Rigobello MP, Paloschi L, Folda A, Moggach SA, Parsons S, Ronconi L, Fregona D, Bindoli A. 2007. Gold(III)-dithiocarbamato complexes induce cancer cell death triggered by thioredoxin redox system inhibition and activation of ERK pathway. Chem Biol 14:1128–1139.

Schumaker PT. 2006. Reactive oxygen species in cancer cells: Live by the sword, die by the sword. Cancer Cell 10:175–176.

Seemuller E, Lupas A, Stock D, Lowe J, Huber R, Baumeister W. 1995. Proteasome from *Thermoplasma acidophilum*: A threonine protease. Science 268:579–582.

Shi G, Chen D, Zhai G, Chen MS, Cui QC, Zhou Q, He B, Dou QP, Jiang G. 2009. The proteasome is a molecular target of environmental toxic organotins. Environ Health Perspect 117:379–386.

Spataro V, Norbury C, Harris AL. 1998. The ubiquitin-proteasome pathway in cancer. Br J Cancer 77:448–455.

Szweda PA, Friguet B, Szweda LI. 2002. Proteolysis, free radicals, and aging. Free Radic Biol Med 33:29–36.

Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160:1–40.

Wang Y, He QY, Sun RW, Che CM, Chiu JF. 2005. GoldIII porphyrin 1a induced apoptosis by mitochondrial death pathways related to reactive oxygen species. Cancer Res 65:11553–11564.

Ward JR. 1988. Role of disease-modifying antirheumatic drugs versus cytotoxic agents in the therapy of rheumatoid arthritis. Am J Med 85:39–44.

Wong E, Giandomenico CM. 1999. Current status of platinum-based antitumor drugs. Chem Rev 99:2451–2466.

Wood DE, Newcomb EW. 2000. Cleavage of Bax enhances its cell death function. Exp Cell Res 256:375–382.

Zhu C, Raber J, Erikson LA. 2005. Hydrolysis process of the second generation platinum-based anticancer drug cis-amminedichlorocyclohexylamineplatinum(II). J Phys Chem B 109:12195–12205.

Zou J, Parkinson JA, Chen Y, Sadler PJ. 1999. Gold(III)-induced oxidation of glycine. Chem Commun 15:1359–1360.