Tubocapsenolide A, a Novel Withanolide, Inhibits Proliferation and Induces Apoptosis in MDA-MB-231 Cells by Thiol Oxidation of Heat Shock Proteins*

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Abstract

Tubocapsenolide A (TA), a novel withanolide-type steroid, exhibits potent cytotoxicity against several human cancer cell lines. In the present study, we observed that treatment of human breast cancer MDA-MB-231 cells with TA led to cell cycle arrest at G1 phase and apoptosis. The actions of TA were correlated with proteasome-dependent degradation of Cdk4, cyclin D1, Raf-1, Akt, and mutant p53, which are heat shock protein 90 (Hsp90) client proteins. TA treatment induced a transient increase in reactive oxygen species and a decrease in the intracellular glutathione contents. Nonreducing SDS-PAGE revealed that TA rapidly and selectively induced thiol oxidation and aggregation of Hsp90 and Hsp70, both in intact cells and in cell-free systems using purified recombinant proteins. Furthermore, TA inhibited the chaperone activity of Hsp90-Hsp70 complex in the luciferase refolding assay. N-Acetylcysteine, a thiol antioxidant, prevented all of the TA-induced effects, including oxidation of heat shock proteins, degradation of Hsp90 client proteins, and apoptosis. In contrast, non-thiol antioxidants (trolox and vitamin C) were ineffective to prevent Hsp90 inhibition and cell death. Taken together, our results demonstrate that the TA inhibits the activity of Hsp90-Hsp70 chaperone complex, at least in part, by a direct thiol oxidation, which in turn leads to the destabilization and depletion of Hsp90 client proteins and thus causes cell cycle arrest and apoptosis in MDA-MB-231 cells. Therefore, TA can be considered as a new type of inhibitor of Hsp90-Hsp70 chaperone complex, which has the potential to be developed as a novel strategy for cancer treatment.

Breast cancer is a major cause of cancer death in women throughout the world. Although hormonal therapy is effective for the treatment of most patients with estrogen receptor-positive breast cancer, resistance to hormonal agents is frequent (1). Chemotherapy is a major treatment modality for hormone refractory breast cancer and estrogen receptor-negative breast cancer. However, women with advanced metastatic breast cancer that is resistant to hormonal therapy usually respond poorly to conventional chemotherapy (2). Therefore, there is an urgent need to develop new effective agents for the treatment of metastatic breast cancer.

Activation of apoptosis pathways is a key mechanism by which anticancer drugs kill tumor cells (3, 4). It is known that anticancer drugs can induce apoptosis signaling through two major pathways (5). One is the mitochondrial (intrinsic) pathway, and the other is the death receptor (extrinsic) pathway. In the mitochondrial pathway, death signals stimulate mitochondria, resulting in the release of proapoptotic factors, such as cytochrome c, from mitochondria (6). The released cytochrome c, together with Apaf-1, activates caspase-9, which in turn proteolytically activates downstream caspase-3, one of the major caspasases participating in the execution phase of apoptosis (6–8). Activated caspase-3 cleaves a lot of substrates, including poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme, and leads to inevitable cell death. In the death receptor pathway, upon interaction with its ligand, such as TNF family ligands, death receptors recruit and activate caspase-8 (9, 10). Caspase-8 can directly activate caspase-3, or it can activate Bid, which then triggers the mitochondrial apoptosis pathway (11).

Plants are a rich source of unique compounds that induce growth inhibition or apoptosis in premalignant or malignant human cells (12, 13). Many compounds used in cancer chemotherapy are derived from plant sources, such as vinca alkaloids, paclitaxel, camptothecin, and etoposide. Withanolides are a group of steroidal lactones found among members of the Solanaceae (14). It was reported that withanolides possess anti-tumor, antiangiogenic, anti-invasive, chemopreventive, and anti-inflammatory activities (15). Therefore, withanolides may represent useful leads for development of potential anticancer drugs. In our previous study, the methanolextract of Tubocapsicum anomalum (Franchet and Savatier) Makino (Solanaceae), which is a native of Taiwan, exhibited significant cytotoxicity against a panel of cancer cell lines (16). Using bioassay-directed fractionation, 17 withanolides were isolated.

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3 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; TA, tubocapsenolide A; H2DCFDA, 2'-7'-dichlorodihydrofluorescein diacetate; Z, benzoyloxycarbonyl; fmk, fluoromethylketone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BSO, DL-buthionine-(SR)-sulfoximine; PBS, phosphate-buffered saline; Hsp, heat shock protein; ROS, reactive oxygen species; NAC, N-acetylcysteine.
Among them, tubocapsenolide A (TA) possessed the most potent activity against the human breast cancer cell lines MDA-MB-231 and MCF-7, the human liver cancer cell lines HepG2 and Hep3B, and the human lung cancer cell line A549, with IC_{50} values less than 1 μg/ml (16). In the present study, the molecular mechanisms of action of TA were investigated in MDA-MB-231 cells, which are estrogen receptor-negative, p53 mutant, and highly metastatic breast cancer cells (17, 18). Our results indicate that TA induces apoptosis and cell cycle arrest at G1 phase in MDA-MB-231 cells, and these effects are related with interference of the chaperone function of heat shock proteins through a thiol oxidation mechanism.

**EXPERIMENTAL PROCEDURES**

**Drugs and Chemicals**—RPMI 1640 medium, fetal bovine serum, and streptomycin/penicillin G were obtained from Invitrogen. 2′,7′-Dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and monochlorobimane were obtained from Molecular Probes, Inc. (Eugene, OR). Anti-caspase 3, anti-caspase 9, anti-cyclin D1, anti-cyclin E, anti-Cdk4, anti-Cdk2, anti-Raf-1, anti-p21, anti-p27, anti-p53, and anti-PARP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-heat shock protein 70 (anti-Hsp70) and anti-Hsp90 antibody, recombinant Hsp70, and recombinant Hsp90-α were purchased from Stressgen Biotechnology Corp. (Victoria, Canada). Anti-Akt, anti-p85, and anti-α-tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA). Benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-fmk), proteasome inhibitor I, MG132 was obtained from Calbiochem. Rabbit reticulocyte lysate and Bright Glo luciferase assay reagent were purchased from Promega. DMSO, calpeptin, NH$_4$Cl, N-acetylcysteine, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, DL-buthionine-(SR)-sulfoximine (BSO), and all other chemicals were obtained from Sigma. TA (Fig. 1A) was isolated from *T. anomalum* (Franchet and Savatier) Makino by the methods described previously (16). The purity of TA used in our study is over 98% determined by high pressure liquid chromatography. The DMSO stock solution was kept at −20 °C and freshly diluted to the desired concentration with culture medium immediately before use (the final concentration of DMSO in culture medium was 0.2%).

**Cell Culture**—MDA-MB-231 human breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA). Cells were propagated in RPMI 1640 medium and supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified 5% CO$_2$ incubator at 37 °C. The cells were harvested by trypsinization and plated 24 h before treatment with the test drugs.

**Evaluation of Cell Viability**—The cell viability was determined by the MTT assay (19). 8 × 10$^3$ cells were plated in 96-well microtiter plates and treated with the various concentrations of TA for different times as indicated. At the end of each time point, 100 μl of MTT (0.5 mg/ml) were added to each well after removing the growth medium. The plates were then incubated at 37 °C for 1 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. At the end of the 1-h incubation, the MTT solution was removed, and 100 μl of DMSO were added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at 550 nm. All of the experiments were plated in triplicate, and the results of assays were presented as means ± S.E. The IC$_{50}$ data were collected from dose–response data of at least four experiments.

**Determination of Apoptotic Cells**—MDA-MB-231 cells (2 × 10$^5$ cells/well) were seeded onto 6-well plates and treated with the test drugs for the indicated times. Harvested cells were fixed overnight in 70% ethanol at 4 °C and then collected by centrifugation and resuspended in phosphate-buffered saline (PBS) containing 25 μg/ml RNase and 0.5% Triton X-100 and incubated for 1 h at 37 °C. Finally, cells were stained with 50 μg/ml propidium iodide for 15 min at 4 °C in the dark. The relative DNA content of these cells were analyzed by a flow cytometer (EPICS XL-MCL; Beckman Coulter) based on red fluorescence. Ten thousand cells were counted, and the histogram of the cell cycle distribution was analyzed by WinMDI 2.8 software. The percentage of cells with hypodiploid DNA content (sub-G$_1$) represented the fraction undergoing apoptotic DNA fragmentation.

**Western Blot Assay**—Following exposure to various drugs, 3 × 10$^6$ MDA-MB-231 cells were washed with PBS and lysed for 10 min at 4 °C in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 μg/ml leupeptin, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, and 5,000 units/ml aprotinin). After the cell suspensions were centrifuged at 16,000 × g for 15 min at 4 °C, the supernatants were collected as the Triton-soluble fraction. The pellet (Triton-insoluble fraction) was lysed in 2% SDS in 50 mM Tris-HCl and boiled for 15 min. The protein concentration of each sample was determined using a Bio-Rad protein assay kit. Equal sample was diluted 1:2 in SDS-sample buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol, 4% bromphenol blue), heated to 95 °C for 5 min, and then loaded onto SDS-polyacrylamide gels. After electrophoresis to nitrocellulose membrane, membranes were blocked in Tris-buffered saline with Tween 20 (TBS-T; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% (w/v) nonfat milk for 1 h at room temperature. ImmunobLOTS were incubated with primary antibodies overnight at 4 °C. Detection was performed using goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase secondary antibody for 1 h at room temperature and an ECL detection system (Amersham Biosciences).

For analysis of disulfide-bonded protein by nonreducing SDS-PAGE, drug-treated cells were washed with PBS at the indicated times and then incubated in ice-cold PBS with 40 mM iodoacetamide (IA) for 5 min to prevent thiol-disulfide exchange and inhibit postlysis oxidation of free cysteines (20). Moreover, sample was diluted in SDS-sample buffer without reducing agents before loading onto SDS-polyacrylamide gels.

**Detection of Intracellular Reactive Oxygen Species Accumulation**—Intracellular reactive oxygen species accumulation was monitored using H$_2$DCFDA, which is a relative specific probe for the presence of hydrogen peroxide (21). At the end of the treatments, cells were loaded with H$_2$DCFDA (10 μM) and incubated at 37 °C for 30 min in the dark. Cells were
**TA Induces Thiol Oxidation of Heat Shock Proteins**

A.

**B.**

**C.**

**D.**

FIGURE 1. *Inhibitory effect of TA on cell viability of MDA-MB-231 human breast cancer cells.* A, chemical structure of TA. B, dose-response curve of TA on cell viability in MDA-MB-231 cells. Cells were treated with DMSO or variant concentrations of TA for 24–48 h. Cell viability was measured by a conventional tetrazolium-based (MTT) assay. C, TA inhibits cell proliferation of MDA-MB-231 cells. Cells were treated with DMSO or TA (3 and 6 μM) for 24–48 h. The total cell number was counted by a hemocytometer. D, TA induces cytotoxicity in MDA-MB-231 cells. The cell viability was determined by the trypan blue exclusion assay. Results are presented as means ± S.E. of 4–6 independent experiments. *, p < 0.05; ***, p < 0.001 as compared with the control.

then collected, washed, and resuspended in PBS and analyzed immediately using flow cytometry with the excitation and emission wavelengths of 490 and 530 nm, respectively.

**Determination of GSH Levels**—The levels of GSH were determined as described previously (22). After treatments, 1 × 10^5 cells in 96-well plates were loaded with 100 μM monochlorobimane (Molecular Probes) for 30 min in the dark. Plates were analyzed immediately using a fluorometer (FLX-800) (360-nm excitation filter and 460-nm emission filter).

**Luciferase Refolding Assay**—Rabbit reticulocyte lysate (RRL) contains abundant quantities of the heat shock proteins, Hsp90 and Hsp70 (23). RRL was treated with DMSO or TA for 30 min at 37 °C. Firefly luciferase (Promega) was inactivated for 40 min at 25 °C in denaturation buffer (30 mM HEPES, pH 7.2, 50 mM KCl, 5 mM MgCl₂, 7 M urea). Then inactivated luciferase was diluted 125-fold into refolding buffer (30 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 1 mM ATP, 10 mM phosphocreatine, and 17.5 units of creatine phosphocreatine) that was supplemented with or without 50% drug-pretreated RRL and incubated at 25 °C. At the indicated times, 5-μl aliquots were removed from the refolding reaction mixture to a white-walled 96-well plate and mixed with 50 μl of Bright Glo luciferase assay reagent (Promega). Luciferase activity was then measured with a luminometer (FLX-800). Native luciferase activity was taken as 100%. The effects of drug treatments on the activity of native luciferase were assayed at 2 h to ensure that TA did not directly inhibit luciferase activity. The chaperone activity of RRL was assayed by measuring luciferase activity following refolding of denatured enzyme (24, 25).

**Statistics**—Data are presented as means ± S.E., and comparisons were made using Student’s *t* test. A probability of 0.05 or less was considered statistically significant.

**RESULTS**

**TA Induces Antiproliferative Effect in MDA-MB-231 Cells**—To analyze the cytotoxicity of TA, MDA-MB-231 cells were incubated with increasing doses of TA (0.03–30 μM) or DMSO (0.2%, vehicle control), and cell viability was determined by a conventional tetrazolium-based (MTT) assay. As shown in Fig. 1B, TA inhibited cell viability of MDA-MB-231 cells in a dose-dependent manner. The IC₅₀ value of TA was 2.7 and 1.8 μM at 24 and 48 h after treatment, respectively. Similar to the MTT assay, the data analyzed by the trypan blue exclusion method also showed that TA potently inhibited cell growth and induced cell death (Fig. 1, C and D).

**TA Induces Caspase-dependent and Caspase-independent Apoptosis in MDA-MB-231 Cells**—To clarify whether TA-induced cell death involved apoptosis, MDA-MB-231 cells were incubated with 3 or 6 μM TA for 24 h, and then the ploidy of DNA was determined by flow cytometry after propidium iodide staining of nuclei. As shown in Fig. 2A, TA induced a dose-dependent increase in the proportion of cells in the sub-G₁ population, suggesting that the cells underwent DNA fragmentation, which is a biochemical hallmark of apoptosis. We next examined the role of caspases in TA-induced apoptosis. Fig. 2B showed that TA-induced proteolytic activation of caspase-8 and caspase-9 in a concentration- and time-dependent manner. In TA-treated cells, cleavages of both procaspase-8 and -9 were significant, observed at 12 h after administration. Caspase-3 is a downstream effector caspase and can be proteolytically activated by both caspase-8 and -9 (26, 27). As expected, TA concentration-dependently induced cleavage of procaspase-3 to an active form (p21/p17). The activation of caspase-3 was further confirmed by the cleavage of PARP, which is a substrate of active caspase-3 (Fig. 2B). When MDA-MB-231 cells were pretreated with Z-VAD-fmk (100 μM), a pancaspase inhibitor, the low concentration (3 μM) of TA-induced apoptosis was almost completely prevented (Fig. 2A). However, Z-VAD-fmk pretreatment did not prevent apoptosis caused by a higher concentration (6 μM) of TA, despite the fact that TA-induced PARP cleavage was completely prevented by Z-VAD-fmk (Fig. 2C). This finding
suggested that caspase activation is not the only mechanism for TA-induced apoptosis and that the caspase-independent pathway also contributes to the TA-induced cell death in MDA-MB-231 cells.

**TA Reduces the Levels of Proteins That Regulate the Mitogenic Signals**—To examine whether TA interfered with cell cycle progression, MDA-MB-231 cells were treated with TA and analyzed by PI staining and flow cytometry. Fig. 3 showed that TA-treated cells accumulated in the G1 phase with a concomitant decrease in the S phase of the cell cycle. We next determined the expression of proteins that involved in the G1 phase of cell cycle control and those regulated cell survival by Western blotting. The decreases of cyclin D1 and Cdk4 protein levels and the increase of hypophosphorylated Rb were observed as early as 1 h after the addition of TA (Fig. 4A). Additionally, the protein levels of Raf-1 and Akt, which can cooperate to promote cell cycle progression (28), were also examined. As shown in Fig. 4A, Raf-1 and Akt protein contents were decreased significantly at 3 h and almost depleted at 12 h after TA treatment. Moreover, the induction of p21 and p27, two important Cdk inhibitors, was detectable after 3 and 6 h of TA treatment, respectively (Fig. 4B). These results suggested that TA induced G1 arrest in MDA-MB-231 cells by down-regulation of cyclin D1 and Cdk4 and by induction of Cdk inhibitors.

**TA-induced Protein Degradation Is Proteasome-dependent**—Protein degradation is induced in various ways, including proteasome- or lysosome-, caspase-, and calpain-dependent pathways. To analyze which pathway was responsible for the degradation of proteins induced by TA, various protease pathway inhibitors, including the proteasome inhibitors MG132 and proteasome inhibitor I (29, 30), the lysosome inhibitor NH4Cl (31), the calpain inhibitor calpeptin (32), and the caspase inhibitor Z-VAD-fmk (33), were employed. All of these inhibitors had no effect on protein expression alone (data not shown). In cells treated with proteasome inhibitors and TA, the Akt protein was lost from the Triton-soluble fraction and accumulated in the Triton-insoluble fraction (Fig. 5). A similar result was observed in Cdk4. In contrast, NH4Cl, calpeptin, and Z-VAD-fmk had little or no effect. These results indicated that TA caused protein aggregation and then induced proteasome-dependent protein degradation.

**TA-induced Responses Were Associated with Hsp90 Inhibition**—Treatment of MDA-MB-231 cells with TA led to degradation of Akt, Raf-1, cyclin D1, and Cdk4, and all of them are known as client proteins of Hsp90 (34). Previously, studies documented that proteasome-mediated degradation is the
common fate of Hsp90 client proteins in cells treated with Hsp90 inhibitors (35, 36). Hence, we supposed that TA may interfere with the Hsp90-containing chaperone complex. To confirm this point, the effects of TA on the protein levels of mutant p53, which is also a Hsp90 client protein, and cyclin E, Cdk2, and phosphatidylinositol 3-kinase p85 subunit, which are non-Hsp90-dependent proteins, were examined. As shown in Fig. 6A, TA induced decline of mutant p53, but not cyclin E, Cdk2, and p85, in MDA-MB-231 cells. These results indicated

FIGURE 4. TA causes the degradation of proteins regulating G1 progression (A) and the induction of CKI, p21, and p27 (B). MDA-MB-231 cells were treated with TA (3 or 6 μM) and harvested at the indicated times. Equal amounts of protein (100 μg/lane) were subjected to SDS-PAGE and analyzed by Western blot. Results are representative of three independent experiments.

FIGURE 5. TA-induced loss of proteins is proteasome-dependent. MDA-MB-231 cells were pretreated with proteasome inhibitors I (PSI; 10 μM), MG132 (10 μM), calpeptin (40 μM), ammonium chloride (20 mM), or Z-VAD-fmk (100 μM) for 1 h, followed by a 6-h exposure to TA (3 μM). Harvested cells were lysed in Triton X-100 buffer, and the Triton X-100-insoluble fraction was resolubilized in 2% SDS. Akt and Cdk4 levels were then analyzed by Western blot. Results are representative of three independent experiments.

FIGURE 6. Effects of TA on the expression of non-Hsp90 client proteins (A) and Hsp90 and Hsp70 (B). MDA-MB-231 cells were treated with various concentrations of TA at the indicated times. Equal amounts of protein (100 μg/lane) were subjected to SDS-PAGE and analyzed by Western blot.

FIGURE 7. Effect of TA on redox state in MDA-MB-231 cells. A, TA induces intracellular reactive oxygen species formation. Cells were treated with TA (3 or 6 μM) for up to 3 h. After treatments, cells were loaded with H$_2$DCFDA (10 μM) and further incubated for another 30 min prior to flow cytometry analysis. B, TA depletes intracellular thiol levels. After treatment of TA for the indicated times, cells were loaded with monochlorobimane (100 μM) and further incubated for another 30 min. Fluorescence intensity was detected by a microplate reading fluorometer. Results are presented as mean ± S.E. of four independent experiments. *, p < 0.05; ***, p < 0.001 as compared with the respective control.
TA Induces Thiol Oxidation of Heat Shock Proteins

that TA-induced protein degradation was selective for Hsp90 client proteins.

It was known that Hsp90 inhibitors induce overexpression of Hsp70; thus, Hsp70 induction has been used as a pharmacodynamic measure of Hsp90 inhibition (37). A Western blotting assay revealed that in TA-treated cells, the overexpression of Hsp70 was induced time-dependently from the early stage after the drug exposure (Fig. 6B). In contrast, there was only a slight increase or no change in the protein levels of Hsp90. These results implied that TA might inhibit the function of Hsp90.

TA Affects Redox States in MDA-MB-231 Cells—The function of several chaperones is tightly regulated by the surrounding redox conditions (38, 39). Thus, we wanted to examine whether TA-induced responses were mediated through inducing oxidative stress. The change of intracellular ROS level in MDA-MB-231 cells after TA treatment was monitored using the fluorescent probe H2DCFDA, and the results are presented in Fig. 7. Treatment of cells with TA resulted in a rapid and transient increase in ROS levels. ROS generation was observed at 1 h and declined at 3 h of the treatment periods. In order to examine the effect of antioxidants on TA-induced ROS formation, MDA-MB-231 cells were pretreated with N-acetylcysteine (NAC) (2 mM), trolox (50 μM), or vitamin C (100 μM) 1 h before stimulation of TA. After 2 h of TA treatment, NAC, trolox, and vitamin C inhibited TA-induced ROS formation by 99.4, 77.6, and 44.2%, respectively. To further confirm whether the redox state in the TA-treated cells was changed, we analyzed the total contents of intracellular GSH by using monochlorobimane, which is essentially nonfluorescent until conjugated with GSH (40–43). Cells treated with or without TA were loaded with monochlorobimane and then analyzed by a microplate reading fluorometer. The effect on the depletion of GSH contents was significant in TA-treated cells and occurred time-dependently (Fig. 7B). These results suggested that TA might disturb intracellular redox balance and elicit oxidative stress.

TA Induces Oxidation of Chaperone Proteins—It was reported that chaperone proteins are cytosolic redox-sensitive proteins and are able to form disulfide bond in response to various oxidative insults (20). Accordingly, we asked whether TA inhibited the Hsp90-containing chaperone complex by inducing oxidative modification. To test this hypothesis, disulfide-bonded Hsp70 and Hsp90 were determined in TA-treated cells by using nonreducing SDS-PAGE. When MDA-MB-231 cells were treated with TA for 3 h, the disulfide-linked Hsp70 and Hsp90 exhibited slower migration and thus appeared as higher molecular weight species under nonreducing conditions (Fig. 8A, left), and this phenomenon was not observed under reducing conditions (data not shown). Moreover, disulfide-

FIGURE 8. TA causes the formation of disulfide-linked Hsp70 and Hsp90 in MDA-MB-231 cells. Cells were treated with TA (3 or 6 μM) (A–C) or diamide (500 μM) (C) for 3 h. In some experiments, NAC (2 mM) and BSO (500 μM) were added 1 and 24 h before the treatment of TA, respectively. Cell lysates were prepared to the Triton-soluble fraction and/or the Triton-insoluble fraction as described under “Experimental Procedures.” The protein levels of the disulfide-linked-Hsp70, Hsp 90 (A and B), or α-tubulin (C) were analyzed by nonreducing SDS-PAGE. Results are representative of three independent experiments. D, cells were treated with TA (3 or 6 μM) in the absence or presence of BSO for 24 h. The proportion of cells in sub-G1, phase was determined with propidium iodide fluorescence by flow cytometry. Results are presented as mean ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 as compared with the respective control.
**TA Induces Thiol Oxidation of Heat Shock Proteins**

A. 

![Western Blot Diagram](image)

**FIGURE 9.** Effects of antioxidants on TA-induced responses in MDA-MB-231 cells. After a 1-h pretreatment with NAC (2 mM), trolox (50 μM), or vitamin C (100 μM), TA was added to cells and further incubated for 3 h (A and E) or 24 h (B–D). A, the protein levels of Hsp70 and Hsp90 client proteins were analyzed by Western blot. B, the proportion of cells in sub-G1 phase was determined with propidium iodide fluorescence by flow cytometry. C, the cell viability was determined by MTT assay. D, the morphologic changes of TA-treated cells were examined with a Zeiss microscope. Magnification is ×400. E, the protein levels of the disulfide-linked Hsp70 in the Triton-insoluble fraction were analyzed by nonreducing SDS-PAGE. Results in B and C are presented as mean ± S.E. of four independent experiments. *p < 0.05; ** p < 0.01; *** p < 0.001 as compared with the respective control.

linked chaperone proteins also accumulated in a dose-dependent manner in the Triton-insoluble fraction (Fig. 8A, right), indicating that those proteins were aggregated and damaged. Pretreatment of MDA-MB-231 cells with NAC, a thiol antioxidant, completely prevented TA-induced oxidation and aggregation of heat shock proteins (Fig. 8A). BSO, an inhibitor of GSH synthesis (44), at a concentration (500 μM) that depleted intracellular GSH contents in MDA-MB-231 cells by 53.9% (data not shown), had no significant effect on inducing thiol oxidation and aggregation of Hsp90-Hsp70 (Fig. 8B). However, BSO in combination with TA led to an enhanced increase in oxidative aggregates of Hsp90-Hsp70 in the Triton-insoluble fraction (Fig. 8B). Furthermore, BSO pretreatment also significantly enhanced TA-induced apoptosis (Fig. 8D). Interestingly, α-tubulin, a thiol-containing protein susceptible to oxidation induced by the thiol oxidant diamide, was not affected by TA (Fig. 8C).

**NAC, but Not Trolox or Vitamin C, Prevents TA-induced Responses on MDA-MB-231 Cells**—We next examined whether the prevention of chaperone protein oxidation by NAC would lead to a reduction in TA-induced responses in MDA-MB-231 cells. As shown in Fig. 9, NAC pretreatment almost completely inhibited TA-induced Hsp70 overexpression and Hsp90 client proteins (Akt, Cdk4, and Raf-1) degradation (Fig. 9A). Furthermore, NAC also prevented the effects of TA on apoptosis, cell viability, and cell morphology (Fig. 9, B–D). In contrast, the antioxidants trolox and vitamin C had no effects on TA-induced induction of Hsp70, degradation of Hsp90 client proteins (Fig. 9A), and oxidation of Hsp70 (Fig. 9E) and Hsp90 (data not shown). Although trolox and vitamin C partially inhibited TA-induced apoptosis, they failed to prevent the loss of cell viability and morphologic change caused by TA (Fig. 9, B–D).

**TA Directly Induces Oxidation of Hsp70 and Hsp90 and Inhibits Chaperone Activity**—To clarify whether the oxidation of Hsp70 and Hsp90 by TA in MDA-MB-231 cells was a direct event, an in vitro study was performed. We incubated recombinant Hsp70 and recombinant Hsp90 with TA for 30 min at 37 °C, respectively, and analyzed the disulfide-bonded proteins by immunoblotting under nonreducing SDS-PAGE. As shown in Fig. 10A, TA induced thiol oxidation of Hsp70 and Hsp90, and these effects were prevented by NAC.

Geldanamycin, a well-documented Hsp90 inhibitor that blocks the ATP-binding site on Hsp90, was compared with TA with respect to effects on cell growth and Hsp oxidation. In the MTT assay, geldanamycin inhibited cell viability of MDA-MB-231 cells by 34.5 and 57.1% at a concentration of 10 μM after 24 and 48 h of incubation, respectively. In addition, geldanamycin (3 μM, 24 h) treatment increased the percentage of cells in G1 phase from 34.2 ± 5.0% to 50.3 ± 1.7% (p < 0.001) while concomitantly decreasing the percentage of cells in S phase from 37.3 ± 1.8 to 7.7 ± 0.7% (p < 0.001) as compared with the control cells. Treatment of MDA-MB-231 cells with 3 and 10 μM geldanamycin led to an increase in the proportion of cells in the sub-G1 population (12.0 ± 0.9 and 15.1 ± 0.5% versus control 2.8 ± 0.4%, respectively; p < 0.001) after a 48-h exposure. These results indicated that geldanamycin exhibited a similar but less potent effect than TA on cell proliferation and apoptosis. However, geldanamycin did not induce thiol oxidation and aggregation of Hsp90-Hsp70 either in a cell-free system (Fig. 10A) or in intact cells (data not shown).
Finally, in order to test whether the Hsp90-Hsp70-based chaperone activity was affected by the oxidation reaction in response to the treatment of TA, the capability of rabbit reticulocyte lysate, which contains high levels of Hsp90 and Hsp70, to refold denatured firefly luciferase was determined (23–25). As shown in Fig. 10B, dilution of urea-denatured luciferase (>99% loss of activity) into refolding buffer that contained the control reticulocyte lysate resulted in recovery of 23.9 and 32.9% activity of the native luciferase after 1 and 2 h of denaturation, respectively. The denatured luciferase did not refold to a native state spontaneously in the absence of reticulocyte lysate. Treatment of reticulocyte lysate with TA significantly inhibited the luciferase refolding in a concentration-dependent manner. In addition, TA had no effect on the activity of native luciferase.

**DISCUSSION**

In the present study, TA potently decreased cell viability of MDA-MB-231 cells; the effect is likely to be the result of induction of apoptosis and cell cycle arrest. In MDA-MB-231 cells, TA caused the activation of caspase-8 and -9 preceding caspase-3 and consequently the cleavage of PARP, suggesting that both death receptor- and mitochondria-mediated apoptosis were involved in TA-induced cytotoxicity. The pan-caspase inhibitor Z-VAD-fmk prevented the apoptosis induced by low, but not high, concentrations of TA, suggesting that caspase-independent apoptosis was also involved in TA-induced cell death. In addition, TA induced 

Exploring the mechanism of TA-induced protein degradation, we found that TA inhibited Hsp90-Hsp70-based chaperone machinery in MDA-MB-231 cells. It is known that heat shock proteins, especially Hsp90 and Hsp70, play a critical role as molecular chaperons maintaining the native conformation of proteins (46, 47). In mammalian cells, Hsp90 forms a multichaperone complex with Hsp70 and other cofactors, which is responsible for folding and stabilizing a specific set of client proteins, such as Raf, Akt, v-Src, Her2, Cdk4, mutant p53, focal adhesion kinase, vascular endothelial growth factor receptor, and telomerase (48). Hsp90 inhibition leads to misfolding and aggregation of client proteins, which results in ubiquitination and proteasome-mediated degradation (35, 49, 50). Since Hsp90-dependent proteins are essential for regulating cell growth, apoptosis, angiogenesis, and metastasis, Hsp90 represents a promising therapeutic target for the treatment of cancer (51, 52). In the present study, there are several
lines of evidence indicating that TA acts as an inhibitor of Hsp90-Hsp70-based chaperone machinery, including the following. 1) TA selectively decreased the protein levels of Hsp90 client proteins (Cdk4, cyclin D1, Raf-1, Akt, and mutant p53) without affecting the non-Hsp90 client proteins. 2) In the presence of proteasome inhibitors, either Akt or Cdk4 accumulated in Triton-insoluble fraction of TA-treated cells, suggesting that TA triggers proteasome-dependent degradation of misfolded/aggregated proteins. 3) Nonreducing SDS-PAGE revealed that TA caused disulfide-linked high molecular weight conformers of Hsp70 or Hsp90 both in Triton-soluble and -insoluble fractions, indicating an oxidative insult of these proteins (4). TA directly inhibited chaperone activity in a Hsp90-Hsp70-rich rabbit reticular lysate system. The results clearly indicated that TA is an inhibitor of Hsp90-Hsp70 chaperone machinery.

It has been reported that either Hsp90 or Hsp70 contains reactive cysteine residues that may oxidize to form intermolecular disulfide bonds and that contribute to formation of high molecular weight conformers (20, 53). Moreover, thiol oxidation of Hsp90 and Hsp70 by sulfhydril oxidants leads to impairment of their chaperone activity (53, 54). In the present study, TA treatment led to an increase in ROS generation and a decrease in GSH levels, implying that TA might induce Hsp90-Hsp70 oxidation by disturbing the balance of the intracellular redox state. However, there are several observations suggesting that TA-induced thiol oxidation of Hsp90-Hsp70 is via a direct and selective effect rather than secondary to oxidative stress. First, TA-induced ROS generation was small and transient, and the nonthiol antioxidants (vitamin C and trolox) were ineffective to prevent Hsp90 inhibition and cell death; thus, it is unlikely that ROS plays an important role in the actions of TA. Second, the low dose (3 μM) of TA elicited significant Hsp90-Hsp70 oxidation but only decreased GSH levels by 14%. Third, BSO alone had no effect on inducing the oxidation and aggregation of Hsp90-Hsp70 but significantly enhanced the oxidative effect of TA on heat shock proteins, indicating that although GSH depletion itself is insufficient to induce oxidative aggregation of heat shock proteins, it does increase the susceptibility of Hsp to oxidative insult by TA. Fourth, TA did not induce thiol oxidation of α-tubulin, which possesses thiol groups susceptible to oxidative stress, revealing that TA-induced protein thiol oxidation was not a general phenomenon. Finally, TA induced thiol oxidation of recombinant Hsp90 and Hsp70 in a cell-free system, and this effect could be reversed by NAC. It has been reported that withanolides, such as withaferin A, possessing an αβ-un satu rated ketone moiety in the A ring react with protein thiol-nucleophiles and form Michael addition adducts (55, 56). Because TA also contains an unsaturated A ring, it is likely that TA reacts with the cysteine residues in Hsp90 and Hsp70 in a similar way, although the precise chemical mechanisms remain to be explored. Nevertheless, our results suggest that TA directly induces oxidative aggregation of Hsp90-Hsp70, which may lead to impairment of the chaperone activity. Based on the fact that TA-induced Hsp oxidation correlates well with its inhibitory effect on cell viability, we suggest that TA-induced growth inhibition and cell death are mediated, at least in part, by thiol oxidation and inhibition of Hsp90-Hsp70.

Most Hsp90 inhibitors developed to date, such as geldanamycin, herbimycin A, radicicol, and the purine derivatives PU3 and PU24FCL, exert their activity by binding to the N-terminal ATP binding pocket and inhibiting Hsp90 ATPase activity (57, 58). Some Hsp90 inhibitors that inhibit Hsp90 by other mechanisms have also been reported. For example, novobiocin is known to bind at the C terminus of Hsp90 (59, 60), whereas the hydroxamic acid analogue histone deacetylase inhibitors have been shown to induce acetylation of Hsp90 and inhibit its chaperone function (61, 62). Although it is still unclear how TA binds to Hsp90, the thiol oxidation mechanism of TA is distinct from other Hsp90 inhibitors. Moreover, a potential advantage of TA is that it also impairs Hsp70 function. A major side effect of current Hsp90 inhibitors is to potently induce Hsp70 by themselves (63, 64). Hsp70 is known to protect from apoptotic cell death, and thus the induction of Hsp70 could limit the efficacy of Hsp90 inhibitors (65–67). In fact, it has been reported that genetic or pharmacological inhibition of Hsp70 induction can potentiate the anti-tumor activity of Hsp90 inhibitors (68, 69). In the case of TA, although Hsp70 induction also occurred in TA-treated cells, the chaperone was oxidized and sequentially formed aggregates, indicating that Hsp70 was damaged by TA. This result suggests that, in comparison with the conventional Hsp90 inhibitors, TA has the advantage of impairing the function of Hsp70 and may thus provide more effective anti-tumor activity.

In conclusion, we have shown that TA represents a new type of inhibitor of Hsp90-Hsp70 chaperone machinery. The mechanism of action of TA for inhibiting chaperone activity is distinct from the conventional Hsp90 inhibitors and involves direct thiol oxidation. Chaperone inhibitors behave as typical multitarget drugs, and our findings highlight the important potential of TA to be used both alone and in combination with other chemotherapeutic drugs for cancer treatment.

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