Tannic Acid Potently Inhibits Tumor Cell Proteasome Activity, Increases p27 and Bax Expression, and Induces G_1 Arrest and Apoptosis[1]

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Abstract

Animal studies have demonstrated that a dietary polyphenol known as tannic acid (TA) exhibits anticarcinogenic activity in chemically induced cancers, although the involved molecular target remains unknown. In addition, proteasome inhibitors have been shown to suppress human tumor growth in nude mice. Most recently, we have reported that ester-bond-containing tea polyphenols are potent proteasome inhibitors *in vitro* and *in vivo*. We have hypothesized that TA, which contains multiple similar gallate moieties linked by ester bonds, should inhibit the proteasome activity. Here, we report that indeed TA potently and specifically inhibits the chymotrypsin-like activity of purified 20S proteasome (IC_{50} = 0.06 μg/ml), 26S proteasome of Jurkat T-cell extracts, and 26S proteasome of living Jurkat cells. Inhibition of the proteasome by TA in Jurkat cells results in accumulation of two natural proteasome substrates, the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the proapoptotic protein Bax, followed by growth arrest in G_1 and induction of apoptotic cell death. Our present study suggests that TA targets and inhibits the proteasome in tumor cells, which may contribute to the previously observed anticarcinogenic activity of TA.

Introduction

Tannins are plant-derived polyphenoic compounds with molecular weights of 500-3000 Da, which can be classified into two groups, hydrolysable and condensed tannins (1–3). The hydrolysable tannins, commonly called TA,[3] contain either gallotannins or ellagictannins. On hydrolysis, gallotannins yield glucose and gallic acid (Fig. 1). TA is widely found in food plants and broadly applied to various industrial food additives (1–3). It is estimated that a person on a balanced diet ingests 1 gm of TA every day in the United States (4).

Recently, it has been shown that TA exerts cancer chemopreventative activity in various animal models (3), *e.g.*, TA was able to suppress skin tumor promotion induced by UV-B radiation in hairless mice by ≤70% (5). In addition, TA dietary intake in low doses can exert a strong dose-dependent chemopreventative activity against spontaneous liver tumor development in C3H male mice by ≤87% (6). Furthermore, it has been shown that TA increased survival rate of BALB/c mice bearing syngeneic tumors by ≤30% (7).

Although the molecular mechanisms responsible for the cancer chemopreventative activity of TA remain unknown, *in vitro* studies have suggested a contribution of the apoptosis-inducing activity of TA. Along this line, TA has been shown to induce either growth arrest (8) or apoptotic death (9). Furthermore, TA induced apoptosis preferably in human oral squamous cell carcinoma and salivary gland tumor cell lines than in normal human gingival fibroblasts, whereas gallic acid, a component unit of TA, showed much weaker selective cytotoxicity (10). However, a conclusive mechanistic target protein responsible for the anticancer property of TA has not been identified.

The 20S proteasome, a multicatalytic complex (700 kDa), constitutes the catalytic component of the ubiquitous proteolytic machinery of the 26S proteasome (11–14). The ubiquitin-proteasome system plays a critical role in the specific degradation of cellular proteins, and two important functions of the proteasome are to promote tumor cell proliferation and to protect tumor cells against apoptosis (11–14). It has been shown that the chymotrypsin-like, but not trypsin-like, activity of the proteasome is associated with tumor cell survival (15, 16). Cell proliferation and cell death regulators have been identified as targets of the ubiquitin/proteasome-mediated degradation pathway, including p53 (17), pRb (18), p21 (19), $p27^{Kip1}$ (20), IκB-α (21), and Bax (22).

Most recently, we have reported that ester bond-containing tea polyphenols, such as (-)-epigallocatechin-3-gallate, potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* (IC_{50} 86–194 nM) and *in vivo* (1–10 μM) at the concentrations found in the serum of green tea drinkers (23). Because TA contains 6 to 9 ester bonds (Ref. 2; Fig. 1), we have hypothesized that TA could also inhibit proteasomal activity. Here, we report that ester-bond containing TA potently and selectively inhibits the chymotrypsin-like activity in purified 20S proteasome, 26S proteasome of Jurkat T-cell extracts, and 26S proteasome in intact Jurkat cells. Furthermore, inhibition of the proteasome by TA in Jurkat T cells is associated with accumulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and proapoptotic protein Bax and is accompanied by induction of G_1 arrest and apoptosis.

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[3] The abbreviations used are: TA, tannic acid; AMC, 7-amido-4-methyl-coumarin; PARP, poly(ADP-Ribose) polymerase.

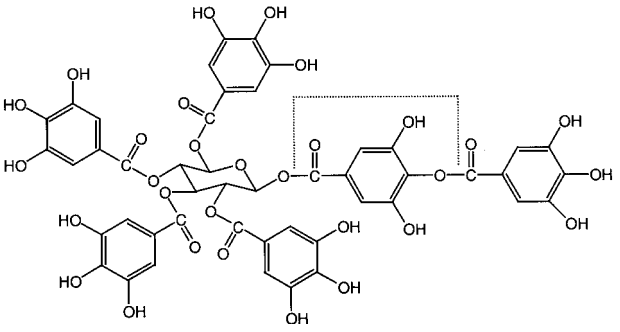


Fig. 1. Structure of gallotannin (TA).

Materials and Methods

Materials. Highly purified TA (gallotannin; ACS Reagent) and D-(+)-glucose (>99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO) and used directly without additional purification. Purified 20S proteasome (*Methanosarcina thermophile*, Recombinant, *Escherichia coli*) and purified calpain I (Human Erythrocytes) were purchased from Calbiochem (La Jolla, CA). Fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) and Suc-Leu-Tyr-AMC (for the calpain I activity) were obtained from Calbiochem, and Z-Gly-Gly-Arg-AMC (for the proteasomal trypsin-like activity) was obtained from Bachem (King of Prussia, PA). The specific calpain inhibitor calpeptin was obtained from Calbiochem. Monoclonal antibody to $p27^{Kip}$ was purchased from PharMingen (San Diego, CA), rabbit polyclonal antibody to human PARP was obtained from Boehringer Mannheim, and human Bax (clone N-20) and actin (clone C11) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture and Cell Extract Preparation. Human Jurkat T cells were cultured in RPMI 1640, supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a 5% CO_2 atmosphere at 37°C. A whole cell extract was prepared as described previously (15). Briefly, cells were harvested, washed with PBS twice, and homogenized in a lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol] for 30 min at 4°C. After that, the lysates were centrifuged, and the supernatants were collected as whole cell extracts.

Inhibition of Purified 20S Proteasome Activity by TA. The chymotrypsin-like activity of purified 20S proteasome was measured as follows. Briefly, 0.5 μg of purified 20S proteasome was incubated with 20 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 30 min at 37°C in 100 μl of assay buffer [20 mM Tris-HCl (pH 8.0)] with or without TA. After incubation, the reaction mixture was diluted to 200 μl with the assay buffer, followed by measurement of the hydrolyzed AMC groups using a VersaFluor Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad).

Inhibition of the Proteasome Activity in Whole Cell Extracts by TA. Jurkat cell extract (6 μg) was incubated for 90 min at 37°C with 20 μM fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC or Z-Gly-Gly-Arg-AMC, for chymotrypsin-like or trypsin-like activities of the proteasome, respectively) in 100 μl of the assay buffer in the presence or absence of TA. The hydrolyzed AMCs were quantified as described above.

Inhibition of the Proteasome Activity in Intact Jurkat T Cells by TA. To measure inhibition of the proteasome activity in living tumor cells, Jurkat T cells (1×10^5 cells/ml/well) were cultured in a 24-well plate. These cells were first incubated for 12 h with various concentrations of TA, followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC. After that, cell medium (200 μl/sample) was collected and used for measurement of free AMCs.

Calpain I Activity Assay. To measure calpain I activity, 3 μg of purified calpain I was incubated with 40 μM fluorogenic peptide calpain substrate, Suc-Leu-Tyr-AMC, for 30 min at 37°C in 100 μl of assay buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM $CaCl_2$, and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, with or without TA or the specific calpain inhibitor calpeptin (24). After incubation, the reaction mixture was diluted to 200 μl with the assay buffer, and the hydrolyzed AMCs were quantified as described above.

Western Blot Analysis. The enhanced chemiluminescence Western Blot analysis was performed using specific antibodies to $p27^{Kip}$, Bax, PARP, or actin, as described previously (15). Briefly, Jurkat T cells were treated with TA for indicated hours (see figure legends), harvested, and lysated in the lysis buffer. Cell lysates (70 μg) were separated by an SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane, followed by enhanced chemiluminescence Western blotting.

Flow Cytometry. Cell cycle analysis based on DNA content was performed as follows. At each time point, cells were harvested, counted, and washed twice with PBS. Cells (5×10^6) were suspended in 0.5 ml of PBS, fixed in 5 ml of 80% ethanol for overnight at −20°C, centrifuged, resuspended again in 1 ml of propidium iodide staining solution (50 μg of propidium iodide, 100 units of RNase A, and 1 mg of glucose per ml PBS), and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is presented as the percentage of cells containing G_1, S, G_2, and M DNA content as judged by propidium iodide staining. Cell death-associated DNA degradation is determined as the percentage of cells containing $<G_1$ DNA content (Pre-G_1).

Results

TA Potently Inhibits the Chymotrypsin-like Activity of Purified 20S Proteasome. Gallotannin is composed of a D-glucose as a core that is linked by six to nine gallate groups through ester bonds (Fig. 1). Because ester bond-containing tea polyphenols are potent proteasome inhibitors (23), we hypothesized that TA would inhibit the proteasome activity. To test this hypothesis, we performed a cell-free proteasome activity assay with or without TA. The result in Fig. 2*A* demonstrates that TA potently inhibited the chymotrypsin-like activity of purified 20S proteasome with an IC_{50} value of 0.06 μg/ml (Fig. 2*A*). The shape of the inhibition curve of TA was similar to that of the specific proteasome inhibitor clastolactacystin β-lactone (Fig. 2*A*, *insert*; Ref. 23), consistent with the conclusion that TA acts as a proteasome inhibitor.

We next determined whether an individual moiety of TA, such as D-glucose or gallate, has any proteasome inhibitory

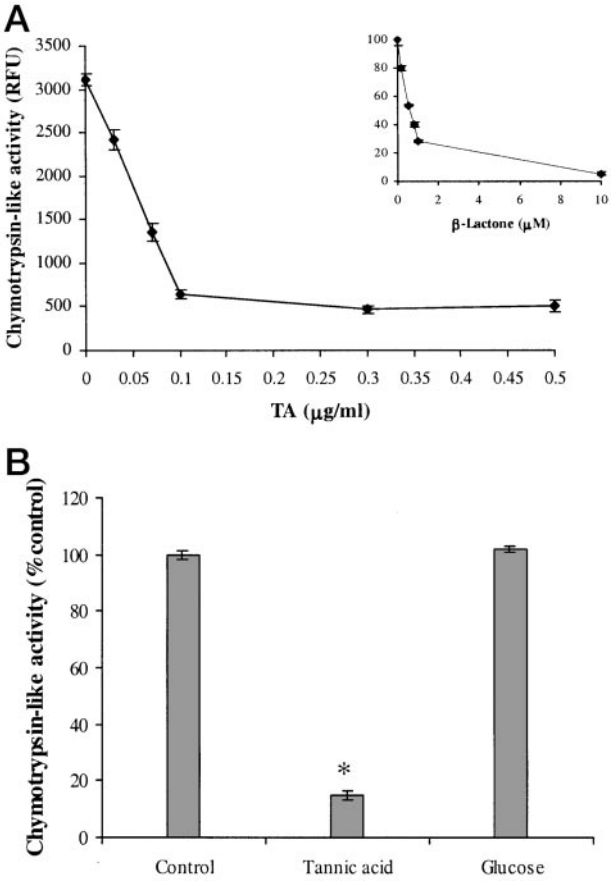


Fig. 2. Inhibition of the purified 20S proteasome activity *in vitro* by TA. In *A*, 0.5 μg of purified 20S proteasome was incubated with 20 μM Suc-Leu-Leu-Val-Tyr-AMC with TA at various concentrations. Inhibitory activity of TA toward the chymotrypsin-like activity of the purified 20S proteasome was measured as described in "Materials and Methods." *Insert*, concentration-dependent inhibition of the chymotrypsin-like activity of the purified 20S proteasome by β-lactone (23). *B*, similar to *A*, effects of TA (0.3 μg/ml) and D-glucose (180 μg/ml) on the chymotrypsin-like activity of the purified 20S proteasome were measured. The values of the error bars are the mean ± SD of three independent experiments. $*P < 0.05$, compared with the control.

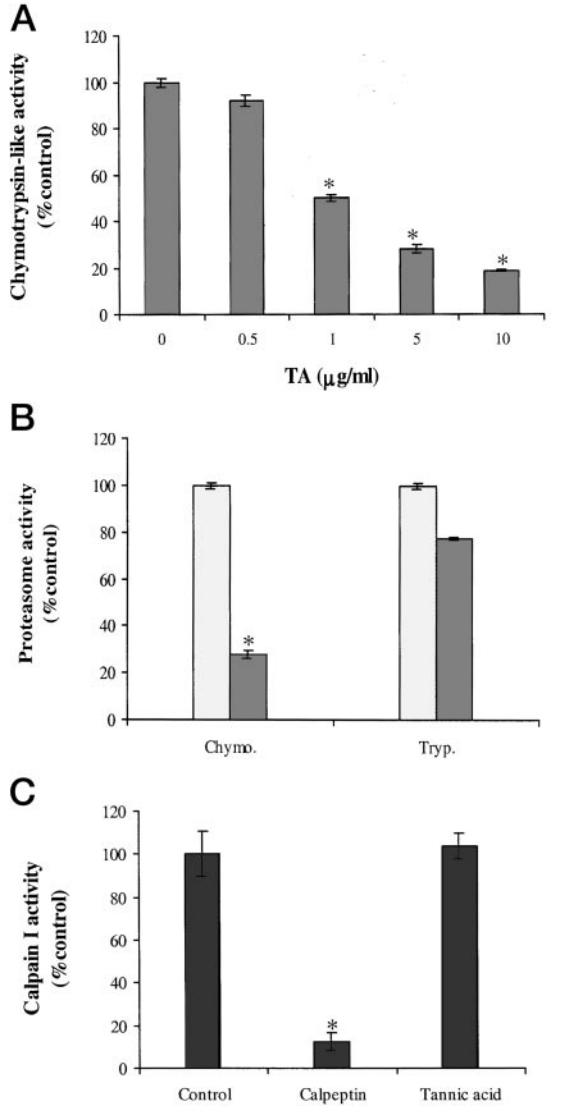


Fig. 3. Selective inhibition of the proteasomal chymotrypsin-like activity by TA. In *A*, Jurkat cell extract (6 μg) was incubated with 20 μM Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity) and TA at indicated concentrations. In *B*, Jurkat cell extract (6 μg) was incubated with 20 μM Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) or Z-Gly-Gly-Arg-AMC (for the proteasomal trypsin-like activity) in the presence of 5 μg/ml TA. In *C*, purified calpain I (3 μg) protein was incubated with 40 μM fluorogenic peptide substrate, Suc-Leu-Tyr-AMC, with the vehicle DMSO (*Control*), TA (5 μg/ml), or the specific calpain inhibitor calpeptin (0.18 μg/ml). After incubation, the hydrolyzed AMCs were quantified as described in Fig. 2. The values of the error bars are the mean ± SD of three independent experiments. $*P < 0.05$, compared with the control.

activity. We found that D-glucose at a very high concentration (180 μg/ml) did not affect the chymotrypsin-like activity of purified 20S proteasome. As a comparison, TA at a 600-fold lower concentration (0.3 μg/ml) inhibited >80% of the 20S proteasomal activity (Fig. 2*B*). In addition, gallate also failed to inhibit the proteasome activity (data not shown and Ref. 23). These results indicate that the ester bonds of TA play an essential role in inhibition of the proteasomal chymotrypsin-like activity.

TA Inhibits the Proteasomal Chymotrypsin-like Activity in Tumor Cell Extracts. We then tested if TA could inhibit the 26S proteasome activity in a tumor cell extract. Protein extract was prepared from exponentially growing human Jurkat T cells and used in the cell-free proteasome activity assay. We found that TA also potently inhibited the proteasomal chymotrypsin-like activity in Jurkat T-cell extract in a concentration-dependent manner: ~50% inhibition at 1 μg/ml and ~80% at 10 μg/ml (Fig. 3*A*).

To study the specificity of TA-mediated inhibition, its effects on the proteasomal trypsin-like and calpain protease activities were then investigated. TA at 5 μg/ml inhibited only 23% of the trypsin-like activity of the proteasome, in contrast to a 73% inhibition of the chymotrypsin-like activity in a Jurkat T-cell extract (Fig. 3*B*). In addition, TA at 5 μg/ml had no inhibitory effects on the purified calpain I activity (Fig. 3*C*), although at 0.06 μg/ml, TA inhibited 50% of the chymotrypsin-like activity of purified 20S proteasome (Fig. 2*A*). As a positive control, the specific calpain inhibitor calpeptin (24) at a 28-fold lower concentration (0.18 μg/ml) inhibited >85% of the purified calpain I activity (Fig. 3*C*). These data suggest that TA preferably inhibits the chymotrypsin-like activity of the proteasome.

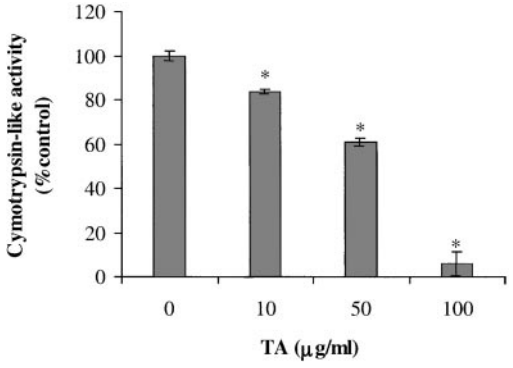


Fig. 4. Inhibition of the chymotrypsin-like activity by TA in intact Jurkat T cells. Intact Jurkat T cells (1×10^5 cells/ml/well) were preincubated for 12 h with various concentrations of TA, followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity). The medium was collected, and the free AMC groups were measured as described in "Materials and Methods." The values of the error bars are the mean ± SD of three independent experiments. $*P < 0.05$, compared with the control.

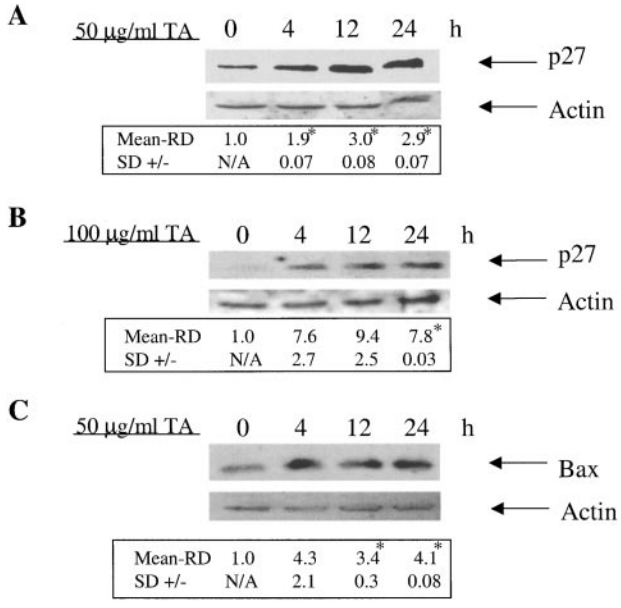


Fig. 5. Accumulation of p27 and Bax proteins in Jurkat T cells treated with TA. Jurkat T cells were treated with 50 (*A* and *C*) or 100 μg/ml (*B*) of TA for the indicated hours, followed by Western blot assay using specific antibodies to p27, Bax (MW 21 kDa), or actin (43 kDa), respectively. RD (relative density) values are normalized ratios of the intensities of p27 or Bax band to the corresponding actin band. The values are the mean ± SD of four independent experiments. $*P < 0.05$, compared with the control.

TA Inhibits the Proteasomal Chymotrypsin-like Activity in Intact Jurkat T Cells. To determine whether TA could also inhibit the living cell proteasomal chymotrypsin-like activity, Jurkat T cells were first incubated with various concentrations of TA, followed by an additional incubation with the fluorogenic proteasome peptide substrate. After that, cell medium was collected for measurement of hydrolyzed products (free AMCs). By performing this assay, we found that TA significantly inhibited the proteasomal chymotrypsin-like activity in intact Jurkat cells in a concentration-dependent manner (Fig. 4). We noticed that the concentrations of TA needed to inhibit the proteasome activity in Jurkat cell extracts (Fig. 3*A*), and intact Jurkat cells (Fig. 4) were much higher than were needed for inhibition of the purified 20S proteasome activity (Fig. 2*A*). However, we have also found that even for a specific proteasome inhibitor, higher concentrations are necessary for inhibition of the living cell proteasome activity (Ref. 23 and see "Discussion").

Accumulation of the Proteasome Target Proteins $p27^{Kip1}$ and Bax in Jurkat T Cells Treated with TA. If TA inhibits the proteasome activity *in vivo*, we would expect to see an increase in levels of proteasome target proteins. To investigate this possibility, Jurkat T cells were treated with TA for ≤24 h, followed by measuring levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the proapoptotic protein Bax, two well-known target proteins of the proteasome (20, 22). Treatment of TA at 50 μg/ml increased p27 levels by 2-fold at 4 h and by 3-fold at 12 and 24 h (Fig. 5*A*). When TA was used at 100 μg/ml, much greater effect was observed; p27 was increased by 8- to 11-fold (Fig. 5*B*). Furthermore, TA at 50 (Fig. 5*C*) or 100 μg/ml (data not shown) also induced Bax expression by 3- to 7-fold. Levels of actin were found to be relatively unchanged during the TA treatment, which was used as a loading control (Fig. 5, *A–C*).

TA Induces G_1 Arrest and Apoptotic Cell Death. It has been documented that p27 acts as an inhibitor of the G_1 to S phase transition (25, 26). If p27 protein accumulated by TA (Fig. 5, *A* and *B*) was functional, the TA-treated tumor cells should exhibit some growth arrest at G_1. To test this possibility, Jurkat T cells were treated with TA under the same conditions described in Fig. 5 and harvested for analysis of cell cycle distribution. Compared with the vehicle-treated cells (Control), treatment with TA at 50 μg/ml increased G_1 population by 5% at 4 h and 15% at 12 h (Fig. 6) before induction of cell death (see below).

Bax has been shown to be an apoptotic cell death promoter (27, 28). We then investigated whether cell death had occurred in TA-treated Jurkat T cells, in association with the increased Bax protein levels (Fig. 5*C*). The first cell death index used was the cell population with $<G_1$ DNA content (indicated by pre-G_1), which measures cell death-associated DNA degradation and can be determined by flow cytometry (15). Another cell death index was the apoptosis-specific cleavage of PARP, which is carried out by activated caspase-3 or -7 and can be measured by Western blotting (27, 28, 22).

Treatment of Jurkat cells with TA at 50 μg/ml for 24 h significantly increased cell death, as judged by a 15% increase in the pre-G_1 cell population (Fig. 6). The p85 PARP cleavage fragment was also detected under the same experimental condition (Fig. 7*A*, *Lane 4*), suggesting induction of apoptotic cell death. At 48 h, cell death was additionally increased, as shown by the 25% increase in the pre-G_1 cell population (Fig. 6) and additional increase in the level of p85/PARP cleavage fragment (Fig. 7*A*, *Lane 5*).

Treatment with TA at 100 μg/ml had greater apoptosis-inducing effect than at 50 μg/ml, because PARP cleavage occurred earlier (at 12 h), and higher levels of p85 PARP cleavage fragment were observed at a fixed time point (12, 24, or 48 h; Fig. 7, *B versus A*). In addition, when Jurkat T cells were treated with various concentrations of TA (10–100 μg/ml) for 24 h, the pre-G_1 cell population was increased in a concentration-dependent manner. Therefore, TA-induced cell death was time- and concentration-dependent.

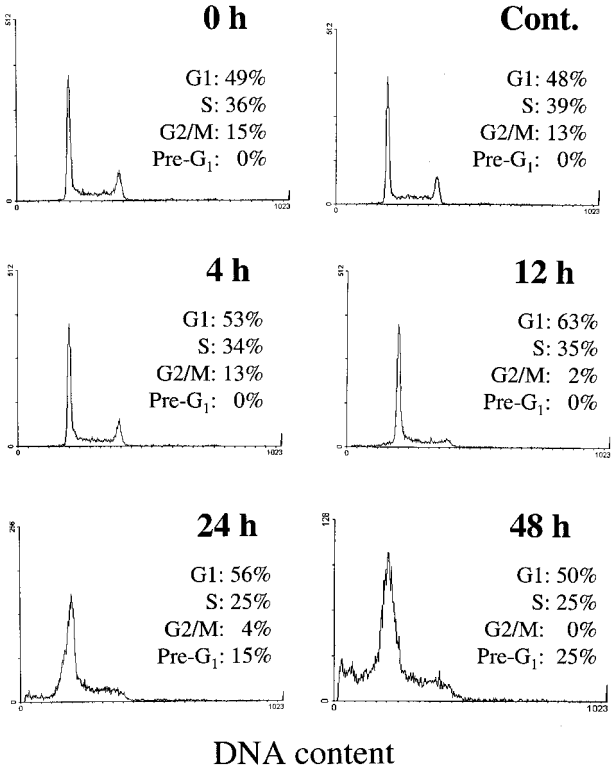
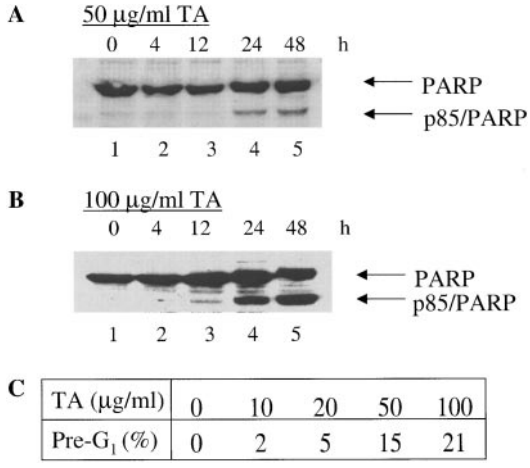


Fig. 6. Induction of G_1 arrest and cell death by TA in Jurkat T cells. Exponentially grown Jurkat T cells (0 h) were treated with 50 μg/ml TA for indicated hours. *Cont.*, control cells treated with the vehicle (H_2O) for 4, 12, 24, or 48 h. All of the control-treated cells exhibited similar cell cycle distribution. At each time point, cells were harvested and analyzed by flow cytometry. Growth arrest is determined by the increase in the percentage of G_1 population, and cell death-associated DNA degradation is measured by the increase in the percentage of cell population with $<G_1$ DNA content (*Pre-G_1*). Similar results were observed in three independent experiments.



C

TA (μg/ml)	0	10	20	50	100
Pre-G_1 (%)	0	2	5	15	21

Fig. 7. TA induces Jurkat cell apoptosis in a concentration-dependent manner. Jurkat T cells were treated with 50 (*A*) or 100 μg/ml (*B*) TA for the indicated hours, followed by Western blot assay using a specific PARP antibody. The intact PARP (116 kDa) and a PARP cleavage fragment (85 kDa) are indicated. In *C*, Jurkat T cells were treated for 24 h with TA at indicated concentrations, followed by flow cytometry. The cell populations with $<G_1$ DNA content (*Pre-G_1*) are shown. Similar results were observed in four independent experiments.

Discussion

Recent animal studies have suggested that TA has a cancer-preventative activity (3, 5–7). Cell culture studies also indicate that TA can induce either growth arrest (8) or apoptosis (9, 10). However, the involved molecular target(s) have not been identified. In the current study, we demonstrated that TA was a potent inhibitor of the proteasomal chymotrypsin-like activity both *in vitro* and *in vivo*. Inhibition of the proteasome activity by TA in intact Jurkat T cells resulted in accumulation of p27 and Bax, associated with G_1 arrest and apoptosis. This finding is consistent with previous reports that show inhibition of the chymotrypsin-like, but not trypsin-like, activity of the proteasome by a specific inhibitor was sufficient to induce either tumor cell growth arrest or apoptosis (15, 16).

It has been shown that the ester bond carbon of β-lactone is responsible for potently and specifically inhibiting the proteasome (29). Our results suggest that ester bonds present in TA are also responsible for its proteasome inhibitory potency. Indeed, each moiety itself of TA, D-glucose, or gallic acid did not inhibit the proteasome activity *in vitro* (Fig. 2*B* and Ref. 23). In addition to the inhibitory potency of TA against the proteasomal chymotrypsin-like activity, the inhibitory specificity of TA was also investigated by testing its effects on other proteasomal or protease activities. TA did not inhibit the activity of purified calpain I, in contrast to being a potent inhibitor of the chymotrypsin-like activity of purified 20S proteasome (Fig. 3*C versus* 2*A*). TA was also much less potent against the proteasomal trypsin-like activity than against the chymotrypsin-like activity in tumor cell extracts (Fig. 3*B*). These results at least suggest that TA preferably inhibits the chymotrypsin-like activity of the proteasome.

When we compared the *in vitro* and *in vivo* potencies of TA, we noted that that ~0.1 μg/ml TA was needed to inhibit ~85% of the chymotrypsin-like activity of purified 20S proteasome (Fig. 2*A*), whereas 10 μg/ml TA was needed for 80% inhibition of the chymotrypsin-like activity in a Jurkat cell extract (Fig. 3*A*), and 50–100 μg/ml TA was needed for a similar inhibitory potency in intact Jurkat T cells (Fig. 4). It suggests that higher concentrations of TA are required for inhibiting cellular proteasome activity *in vivo*. This argument is in agreement with the fact that higher concentrations of other proteasome inhibitors, even specific ones, are also needed to inhibit the proteasome in cells, *e.g.*, the IC_{50} value of the specific proteasome inhibitor β-lactone to inhibit the chymotrypsin-like activity of purified 20S proteasome was 0.1–0.6 μM (23, 29). However, when used in intact Jurkat T cells, β-lactone at 10 μM inhibited only 20% of the proteasomal chymotrypsin-like activity (23). Also, the IC_{50} value of the proteasome inhibitor LLnL to inhibit a purified 20S proteasomal chymotrypsin-like activity was 0.14 μM (30), but 10 μM inhibited only 40% of chymotrypsin-like activity in living Jurkat cells (23). Furthermore, we and other researchers also reported that concentrations of dipeptidyl proteasome inhibitors to inhibit purified 20S proteasome were ~500 times lower than those to inhibit the living cell proteasome activity (15, 31). Finally, tea polyphenol (-)-epigallocatechin-3-gallate showed greater potencies to purified 20S proteasome (IC_{50} 86 nM) than to intact cellular proteasome activity (24% inhibition at 10 μM; Ref. 23).

The concentrations (10–100 μg/ml) of TA we used in Jurkat T cells are similar to those other researchers used in various cell culture systems, *e.g.*, TA at 50–200 μg/ml concentration was shown to be able to inhibit human immunodeficiency virus promoter activity induced by 12-*O*-tetra de-

canoylphorbol-13-acetate in Jurkat T cells (32). In addition, TA at a concentration between 12.5 and 50 μg/ml suppressed 50% of cell growth of isolated human malignant tumors (8). The physiological levels of TA in human or animal bodies are currently unknown. Nepka *et al.* (6) reported that by feeding C3H male mice bearing hepatoma with TA-containing drinking water, TA at 75, 150, and 300 mg/l (or μg/ml) exerted chemopreventative activity. These TA concentrations that exhibited chemopreventative activity exceeded those used in our cell culture experiments. More work is needed in this area to determine the physiological serum concentrations of TA after dietary intake.

The accumulation of p27 and Bax proteins in Jurkat T cells (Fig. 5) was attributable to inhibition of the proteasome activity by TA, which is supported by the following evidence: (*a*) as discussed above, TA is a relatively specific, potent proteasome inhibitor *in vitro* (Figs. 2 and 3); (*b*) TA inhibits the chymotrypsin-like activity of the proteasome *in vivo* (Fig. 4); and (*c*) accumulation of both p27 and Bax proteins was observed in both a time- and concentration-dependent manner (Fig. 5 and data not shown).

The following arguments are consistent with the idea that TA-accumulated p27 and Bax proteins are functional in Jurkat tumor cells. First, when Jurkat T cells were treated with TA, both p27 expression and G_1 population were increased simultaneously in a time-dependent manner (Figs. 5 and 6). This result is also consistent with previous reports that overexpression of p27 could cause growth arrest in G_1 (25, 26). Second, after Bax accumulation (at 4 h; Fig. 5*C*), cell death occurred (at 12 h), as judged by increased levels of pre-G_1 cell population and PARP cleavage (Figs. 6 and 7). TA-induced apoptotic cell death is also time- and concentration-dependent (Figs. 6 and 7). Therefore, accumulation of Bax by TA before apoptosis is consistent with the fact that Bax acts as a cell death promoter (27, 28).

In summery, our current study has demonstrated that TA can inhibit the proteasome activity *in vitro* and *in vivo* and indicated that inhibition of the proteasome activity by TA may be a novel mechanism for its previously observed anticarcinogenic activity (3, 5–7). These studies suggest the importance of plant foods in a cancer preventative diet.

Acknowledgments

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