

Growth Inhibition and Induction of Apoptosis in NB4 Promyelocytic Leukemia Cells by Trypsin Inhibitor from Sweet Potato Storage Roots

GUAN-JHONG HUANG,[†] MING-JYH SHEU,[‡] HSIEN-JUNG CHEN,[§]
YUAN-SHIUN CHANG,[†] AND YAW-HUEI LIN^{*,#}

Institute of Chinese Pharmaceutical Sciences and Department of Physiology, School of Medicine, China Medical University, Taichung 404, Taiwan; Department of Horticulture, Chinese Culture University, Taipei 111, Taiwan; and Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 115, Taiwan

The objective of this study was to investigate the antiproliferative effect and the mechanism of trypsin inhibitor (TI) from sweet potato [*Ipomoea batatas* (L.) Lam. 'Tainong 57'] storage roots on NB4 promyelocytic leukemia cells. The results showed that TI inhibited cellular growth of NB4 promyelocytic leukemia cells in a time-dependent and dose-dependent manner, and treatment for 72 h induced a marked inhibition of cellular growth, showing an IC₅₀ of 57.1 ± 8.26 μg/mL. TI caused cell cycle arrest at the G1 phase as determined by flow cytometric analysis and apoptosis as shown by DNA laddering. TI-induced cell apoptosis involved p53, Bcl-2, Bax, and cytochrome *c* protein in NB4 cells. P53 and Bax proteins were accumulated, and antiapoptotic molecule Bcl-2 was decreased in the tested cells in a time-dependent manner during TI treatment. TI also induced a substantial release of cytochrome *c* from the mitochondria into the cytosol. Hence, TI induced apoptosis in NB4 cells through a mitochondria-dependent pathway, which was associated with the activation of caspase-3 and -8. These results demonstrated that TI induces NB4 cell apoptosis through the inhibition of cell growth and the activation of the pathway of caspase-3 and -8 cascades.

KEYWORDS: Sweet potato; trypsin inhibitor; apoptosis; NB4; caspase

INTRODUCTION

Apoptosis is important in the control of cell numbers during development and proliferation. The mechanism of apoptosis is conserved from lower eukaryotes to mammals and exhibits a network of tightly ordered molecular events that finally converge into the enzymatic fragmentation of chromosomal DNA, driving a cell to death (1). Apoptosis involves the activation of a family of caspases, which cleave a variety of cellular substrates that contribute to detrimental biochemical and morphological changes (2, 3). At least two pathways of caspase activation for apoptosis induction have been characterized. One is mediated by death receptors (Fas). Activation of death receptors by binding with their natural ligands (Fas ligand) induces apoptosis in sensitive cells (4). Death receptor ligands characteristically initiate signaling via receptor oligomerization and recruitment of specialized adaptor proteins followed by proteolysis and activation of procaspase-8, and activated caspase-8 directly cleaves and activates caspase-3, which in turn cleaves other caspases

(e.g., caspase-6 and -7) for activation (5–8). The other pathway, driven by Bcl-2 family proteins, which may be antiapoptotic (Bcl-2 and Bcl-X_L) or proapoptotic (Bax, Bak, and Bid), regulates cell death by controlling mitochondrial membrane permeability during apoptosis. Upon apoptosis, proapoptotic proteins translocate to mitochondria and accelerate the opening of the mitochondria porin channel that leads to cytochrome *c* release, thereby triggering the cascade of caspase activation (9). The induction of apoptosis by the natural products in malignant cells validates a promising strategy for human cancer chemoprevention (10, 11).

Protease inhibitors in plants may be important in regulating and controlling endogenous proteases and in acting as protective agents against insect and/or microbial proteases (12, 13). Sohnie and Bhandarker (14) reported for the first time the presence of trypsin inhibitor (TI) in sweet potato (SP). Later, it was indicated that TI activities in SP are positively correlated with concentrations of water-soluble protein (15) and that TI activities increase in response to drought (16). Polyamines, including cadaverine, spermidine, and spermine, were bound covalently to SP TI, which might participate in regulating the growth and developmental processes of SP (17). TI in SP storage roots accounts for about 60% of the total water-soluble proteins and could be recognized as storage proteins (15). Maeshima et

* Corresponding author [fax 886 (2) 2782-7954; telephone 886 (2) 2789-9590, ext. 321; e-mail boyhlin@gate.sinica.edu.tw].

[†] Institute of Chinese Pharmaceutical Sciences, China Medical University.

[‡] Department of Physiology, China Medical University.

[§] Chinese Culture University.

[#] Academia Sinica.

al. (18) identified sporamin as the major storage protein in SP root, accounting for 80% of the total proteins there; a dramatic decrease of the amount of sporamin to 2% of the original value was found during sprouting. Lin (19) considered sporamin as one form of TI in SP, a finding confirmed later by Yeh et al. (20). However, few papers concern the degradation of SP root storage protein during sprouting. An aspartic protease that could degrade TI in sprouts of sweet potato was purified (21).

Our previous papers showed that TI exhibited both dehydroascorbate reductase and monodehydroascorbate reductase activities, and 33 kDa TI exhibited antioxidant activities against different radicals (22). In this work we report for the first time that TI, the major storage protein of sweet potato tubers, showed cytotoxicity via cell arrest at the G1 phase of the cell cycle and induction of apoptosis of human NB4 leukemia cells. We propose that TI of sweet potato may be useful as a cancer-preventive substance.

MATERIALS AND METHODS

Cultures and Chemicals. NB4 promyelocytic leukemia cell line was purchased from the American Type Culture Collection (Rockville, MD). Tris and electrophoretic reagents were purchased from E. Merck Inc. (Darmstadt, Germany); Seebie prestained markers for SDS-PAGE including myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamate dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa), and insulin B chain (4 kDa) were from Invitrogen (Groningen, The Netherlands); p53, Bcl-2, Bax, cytochrome *c*, and β -actin were from ABCam (ABCam Inc.). Caspase-3 and -8 colorimetric activity assay kits were from Chemicon International, Inc. (Temecula, CA); Coomassie brilliant blue G-250 and other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant Materials. Fresh storage roots of sweet potato (*Ipomoea batatas* Lam. 'Tainong 57') were purchased from a local market. Samples were washed and peeled and then cut into strips that were extracted immediately.

Purification of Sweet Potato Trypsin Inhibitors. Extraction and purification of TI from sweet potato storage roots was carried out at 4 °C according to the method of Hou et al. (22). The storage roots were cut into strips that were extracted immediately with 4 volumes (w/v) of 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl, 1% (w/v) ascorbate, and 1% (w/v) polyvinylpyrrolidone (PVPP) in a homogenizer for 30 s (four times). The homogenates were filtered through four layers of cheesecloth and centrifuged twice at 12000g for 30 min. The crude extracts were loaded directly onto a trypsin-Sepharose 4B affinity column (1.0 × 10 cm), and the adsorbed TI was eluted by changing the pH value with 0.2 M KCl buffer (pH 2.0) (23). The extracts were desalted and concentrated with Centricon 10 and then lyophilized for further use.

Trypsin Inhibitor Activity Assay. Trypsin inhibitor activity (TIA) was assayed according to the method of Huang et al. (24) using *N*-benzoyl-L-arginine ethyl ester as a substrate. Different amounts of TI were preincubated with 4 μ g of trypsin at room temperature for 15 min, and then the substrate was added for an additional 20 min. The absorbance at 405 nm was determined. Three determinations were averaged for TIA and expressed as micrograms of trypsin inhibited. Statistical significance of the data was tested using a commercial SAS program (SAS 8.0, Cary, NC).

Protein Staining and Activity Stainings of TI on 15% Denaturing Polyacrylamide Gels. TI was detected for both protein and activity stainings on 15% SDS-PAGE gels. Samples were mixed with sample buffer, namely, 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with or without 2-mercaptoethanol. Coomassie brilliant blue G-250 was used for protein staining (24). For TI activity staining, the gel was stained according to the method of Hou et al. (25). When SDS-PAGE was finished, gel was immersed and shaken twice in 25% v/v isopropanol in 10 mM Tris buffer (pH 7.9) for 10 min each. The gel was then dipped into 10 mM

hydrogen peroxide in the same buffer for at least 30 min with gentle shaking and finally washed in 10 mM Tris buffer (pH 7.9) for 10 min. Then the gel was incubated in trypsin solution (50 μ g/mL bovine trypsin/mL, 10 mM Tris buffer pH 7.9) at 37 °C for 20 min. After removal of excess trypsin by rinsing with the same buffer, the gel was incubated in the dark at 37 °C for at least 30 min with 160 mL of substrate-dye solution immediately prepared before use. The substrate-dye solution consisted of 40 mg of *N*-acetyl-DL-phenylalanine β -naphthyl ester (APNE) in 16 mL of *N,N*-dimethylformamide that was brought to 160 mL with 144 mL of 10 mM Tris buffer (pH 7.9), in which 80 mg of tetrazotized *o*-dianisidine was dissolved. The gel was destained with 10% acetic acid for 30 min.

Measurement of Cell Proliferation. The NB4 cell line was isolated from long-term cultures of leukemia blast cells on bone-marrow stromal fibroblasts as reported by Lanotte et al. (26). NB4 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% carbon dioxide. The cells were subcultured every third day, maintaining the cell density in cultures below 5×10^5 cells/mL.

Cell Viability Assay. The cell viability assay was determined by using a colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for cell survival and proliferation (Chemicon). Suspensions of human histiolytic lymphoma NB4 monocytes (2×10^5 cells/mL) were cultured with or without test samples (at various concentrations in 10 μ L of suspension) in a 96-well microplate (90 μ L suspension/well). After 24, 48, or 72 h, 10 μ L of MTT solution was added to each well, and the cells were incubated at 37 °C for 4 h. Then, 100 μ L of lysis buffer was added to each well, and the cells were again incubated at 37 °C for 1 h to dissolve the dark blue crystals. Each well was completely pipetted, and then the absorption at 570 nm of formazan solution was measured using a microplate reader. At least three repeats for each sample were used to determine the cell proliferation. The decolorization was plotted against the concentration of the sample extract, and the EC₅₀, which is the amount of test sample necessary to decrease 50% of the absorbance of MTT, was calculated.

Cell Cycle Analysis. NB4 cells (1×10^6) were suspended in hypotonic solution [0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, and 0.1 mM EDTA] and stained with 50 μ g/mL of propidium iodide (PI). DNA content was analyzed by a FACScan (Becton Dickinson, San Jose, CA). The population of cells in each cell cycle phase was determined using CellQuest PRO software (Becton Dickinson).

Assays for Apoptosis. Apoptosis was determined by the presence of internucleosomal DNA fragmentation (DNA laddering) after cell exposure to TI (10, 50, and 100 μ g/mL) in 72 h. NB4 cells were cultured in 24-well microtiter plates at a density of 2×10^6 cells/well (1 mL final volume). To extract genomic DNA, cells were harvested, washed with cold 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM EDTA, and lysed by the addition of 0.5% SDS. Cell lysates were then incubated at 56 °C for 3 h in the presence of 100 μ g/mL of proteinase K. DNA was purified by successive phenol/chloroform extractions, and the resultant aqueous phase was mixed with 3 M sodium acetate (pH 5.2) and absolute ethanol. The mixture was incubated at -20 °C overnight, and the ethanol-precipitated DNA was washed with 70% ethanol. Purified DNA was resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and treated with 50 μ g/mL DNase-free RNase A for 1 h. Samples were resolved on a 1% agarose gel and stained with 0.5 μ g/mL ethidium bromide, and DNA was visualized with ultraviolet light.

Caspase-3 or -8 Activity Assay. A caspase-3 or -8 colorimetric activity assay kit (Chemicon International, Inc.) was used. TI (100 μ g/mL) treated NB4 cells (1×10^6) were washed in PBS and resuspended in 100 μ L of ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 0.03% (v/v) nonidet P-40, 1 mM dithiothreitol] for 30 min at 4 °C. Cell extracts were centrifuged (13000g for 15 min at 4 °C), and aliquots of the supernatants (20 μ g of proteins) were incubated for 30 min at 37 °C with caspase-3 or -8 substrate. Enzymatic caspase activity was calculated from the increase of absorbance at 405 nm during 2 h. The activity data are expressed relative to the blank data.

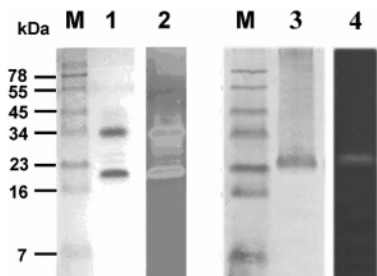


Figure 1. Protein (lanes 1 and 3) and activity (lanes 2 and 4) stainings of the trypsin inhibitor from sweet potato storage root on SDS-PAGE gels without (lanes 1 and 2) or with (lanes 3 and 4) 2-mercaptoethanol. The gel system contained a 2.5 cm, 4% stacking gel and a 4.5 cm, 15% separating gel. M indicates the Seebule prestained markers of SDS-PAGE. Ten micrograms of TI was loaded in each well.

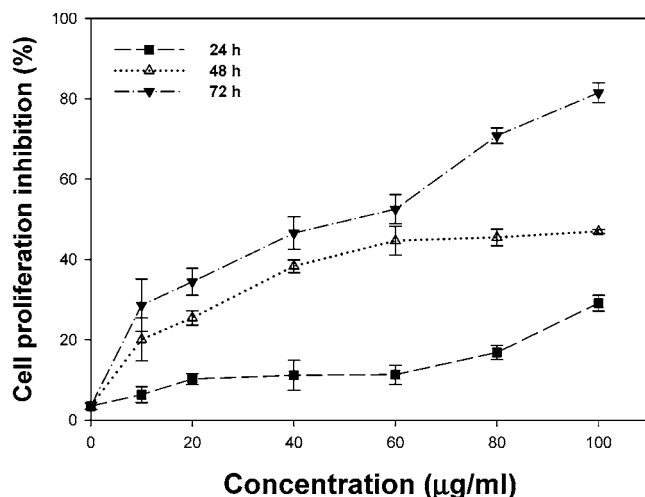


Figure 2. Percent inhibition of NB4 cell proliferation by TI at different concentrations (mean \pm SD, $n = 3$) ($p < 0.05$). After treatment with 10–100 $\mu\text{g/mL}$ TI for 0, 24, 48, or 72 h, cell density of NB4 cells was determined. Cell density was scored in a cytometric chamber, and metabolic activity was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test. Absorbance at 570 nm was recorded using an ELISA plate reader. Cells were seeded at a density of 2×10^5 cells/mL. Data were presented as the mean \pm SEM, determined from three separate experiments.

Cell Lysate Preparation and Western Blotting. Cells were collected by centrifugation at 700g for 10 min, and then the pellets were resuspended in a lysis buffer [1% NP-40, 1 mM phenylmethanesulfonyl fluoride (PMSF), 40 mM Tris-HCl (pH 8.0), 150 mM NaCl] at 4 °C for 15 min. Cell lysates (15 μg of protein per lane) were fractionated on 12.5 or 7.5% SDS-polyacrylamide gels prior to being transferred to the membrane (Immobilon-P membranes, Millipore, Bedford, MA) according to the standard protocol. Membranes were blocked for 2 h at room temperature in 5% nonfat dry milk powder and then incubated with primary antibodies. After incubation, membranes were washed in phosphate-buffered saline with 0.05% Tween (PBST) three times, 10 min each, and then incubated with anti-rabbit alkaline phosphatase-conjugated antibody, washed in PBST three times, 10 min each, and developed using nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma). The second antigen (goat against rabbit Fc portion of Ig) was a product of Sigma. β -Actin was used as an indicator to establish equality of lane loading. Blots were also stained with Coomassie brilliant blue to confirm that equal amounts of protein extract were present in each lane. The following antibodies were used in this study: anti-cytochrome *c*, -p53, -Bax, -Bcl-2, and β -actin (ABcam).

Statistical Analysis. Means of triplicates were measured. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$.

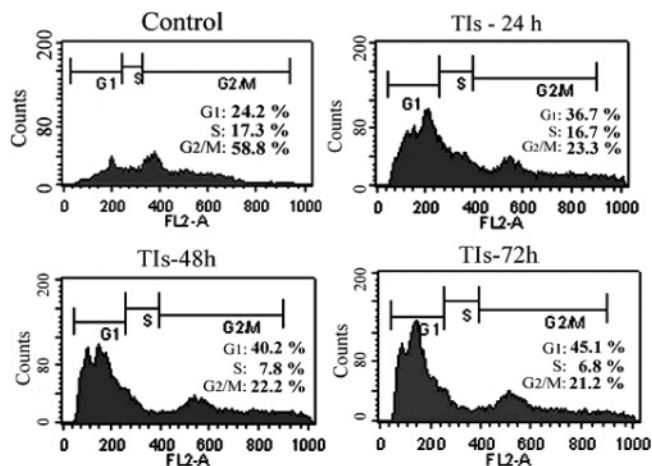


Figure 3. Cell cycle analysis. Cells were cultured with 100 $\mu\text{g/mL}$ TI for 0–72 h and stained with propidium iodide (PI). PI-stained DNA content was analyzed by flow cytometry. G₀/G₁, S, and G₂/M indicate the cell phase. FL2-A represents the intensity of the fluorescence. Data shown were from a representative experiment repeated three times with similar results.

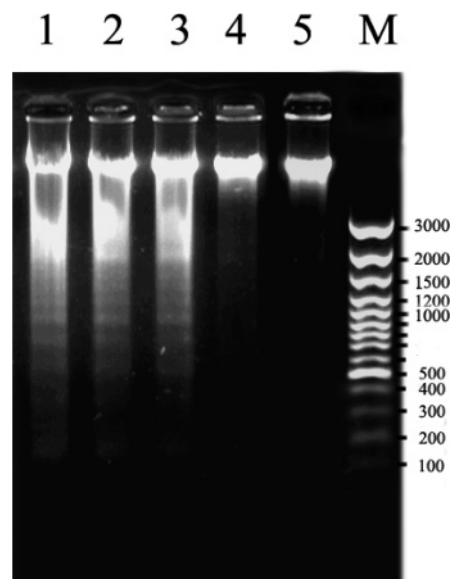


Figure 4. Purified DNA was resuspended in 10 mM Tris-HCl buffer, pH 7.5, with 1 mM EDTA. Lanes 1–5 correspond to NB4 cells with the following treatments: lane 1, TI 10 $\mu\text{g/mL}$; lane 2, TI 50 $\mu\text{g/mL}$; lane 3, TI 100 $\mu\text{g/mL}$; lane 4, control (phosphate-buffered saline); lane 5, control (no treatment). Samples were resolved on a 1% agarose gel and stained with 0.5 $\mu\text{g/mL}$ ethidium bromide, and DNA was visualized with ultraviolet light. M, 100 bp DNA ladder.

RESULTS AND DISCUSSION

Extraction and Purification of Trypsin Inhibitor from Sweet Potato Storage Root. TI was purified from sweet potato storage root according to the method of Hou et al. (22). **Figure 1** shows protein staining (lanes 1 and 3) and activity staining (lanes 2 and 4) of purified TI without (lanes 1 and 2) or with (lanes 3 and 4) 2-mercaptoethanol treatment on a 15% SDS-PAGE gel.

TI Inhibited Cellular Proliferation of NB4 Cells via Induction of G₁ Cell Cycle Arrest and Apoptosis. TI (0, 10, 20, 40, 60, 80, and 100 $\mu\text{g/mL}$) inhibited cellular growth of NB4 promyelocytic leukemia cells in a time-dependent and dose-dependent manner (**Figure 2**), and treatment for 72 h

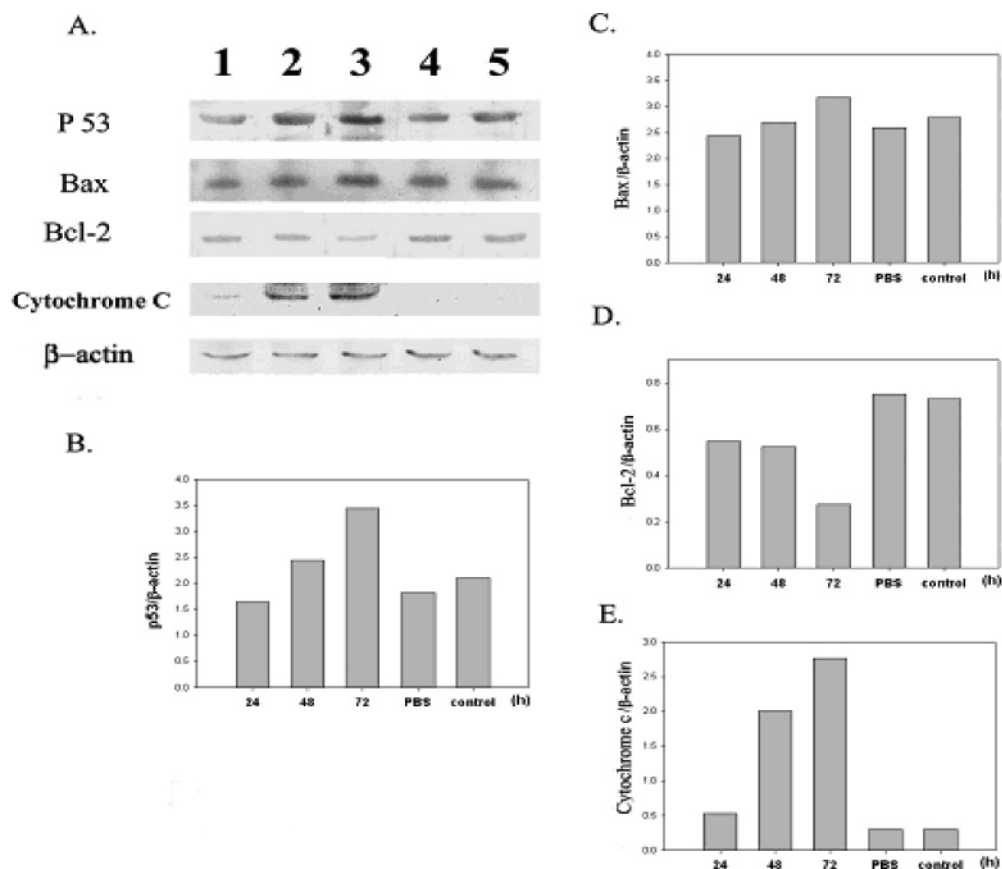


Figure 5. Expression of apoptosis- and cell cycle-associated proteins. (A) NB4 cells were treated with 100 $\mu\text{g}/\text{mL}$ TI for the indicated times. Cell lysates (15 μg per lane) were fractionated on 10% SDS–polyacrylamide gels and analyzed by Western blotting with antibodies against apoptosis- and cell cycle-associated proteins (p53, Bcl-2, Bax, cytochrome *c*, and β -actin): lane 1, treated with TI for 24 h; lane 2, treated with TI for 48 h; lane 3, treated with TI for 72 h; lane 4, control (phosphate-buffered saline); lane 5, control (no treatment). (B) P53/ β -actin protein ratios, (C) Bax/ β -actin protein ratios, (D) Bcl-2/ β -actin protein ratios, and (E) cytochrome *c*/ β -actin protein ratios were obtained by treating NB4 cells with TI for various times.

induced a marked inhibition of cellular growth, showing an IC_{50} of $57.1 \pm 8.26 \mu\text{g}/\text{mL}$ (Figure 2).

The apoptotic effects of TI were confirmed by flow cytometric analysis. Cells were analyzed for cell cycle distribution by flow cytometry. Typical morphological appearance of apoptosis was observed, including condensed chromatin and fragmented nuclei with apoptotic bodies (data not shown). Interestingly, cultivation with TI strongly increased the population of NB4 cells in the G1 phase 72 h after treatment (Figure 3). When NB4 cells were incubated with 100 $\mu\text{g}/\text{mL}$ of TI for 0, 24, 48, and 72 h, the relative percentages of G1 phase were 24.2, 36.7, 40.2, and 45.1%, respectively. These results indicate that TI caused cell cycle arrest at the G1 phase, followed by apoptosis.

Effect of TI on Nuclear DNA Fragmentation in NB4 Cells.

One of the mechanisms by which cell growth is suppressed is apoptotic cell death. Therefore, the effect of TI on DNA fragmentation was examined in NB4 cells (Figure 4). Nucleosomal DNA fragmentation was observed when cells were treated with 10, 50, and 100 $\mu\text{g}/\text{mL}$ of TI for 72 h. The profile for TI-induced apoptosis closely correlated with its growth-suppressive effects. Thus, the growth suppression induced by TI in NB4 cells may be related to the induction of apoptosis.

Expression of Cell Cycle-Associated Proteins in TI-Treated NB4 Cells. To understand the molecular mechanisms by which the TI induced apoptosis, we examined various apoptosis-related proteins. NB4 cells were cultured in media containing 100 $\mu\text{g}/\text{mL}$ of TI for 0, 24, 48, and 72 h. At each time point, total proteins were isolated and p53, Bcl-2, Bax,

cytochrome *c*, and β -actin immunoreactivity levels were measured by Western blotting (Figure 5). P53 protein was accumulated during the treatment of TI in NB4 cells expressing wild-type p53 (Figure 5). The proapoptotic protein Bax was increased, whereas the levels of the antiapoptotic molecule Bcl-2 were decreased in a time-dependent manner during the treatment.

TI also induced a substantial release of cytochrome *c* from the mitochondria into the cytosol (Figure 5). These results suggest that TI induces apoptosis in NB4 cells through a mitochondria-dependent pathway.

Apoptosis may be initiated by many different signals, from either within or outside the cell. During the modulation phase of apoptosis many different genes such as *p53*, *c-myc*, or *Bcl-2/Bax* have been shown to be able to shift the balance between cellular proliferation or stationary state and death (27–29).

The tumor suppressor protein p53, a transcription factor, has been identified as a participant in the cellular DNA damage response. Upon DNA damage, p53 up-regulation causes G1 arrest. The apoptosis-promoting capacity of p53 is presumably due to its ability to activate *bax*, a gene that encodes an inhibitor of Bcl-2. In the present study, we observed down-regulation of *bcl-2* expression, which is in keeping with the role of Bcl-2 in blocking apoptosis and promoting proliferation. At the same time, the expression of apoptosis-associated gene *p53* was up-regulated. These findings suggested that Bcl-2, Bax, and p53 were involved in apoptosis of NB4 cells induced by TI.

Many papers have pointed out that the ability to induce apoptosis on tumor cells by anticancer agents (such as Taxol)

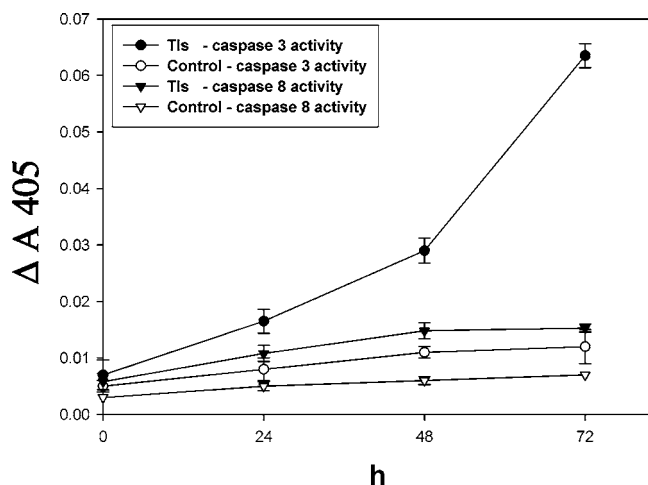


Figure 6. Time course of caspase-3 or -8 activity induced by TI. A caspase-3 or -8 colorimetric activity assay kit was used. NB4 cells were cultured in 96-well microtiter plates at 1×10^6 cells/well and treated with TI 100 $\mu\text{g/mL}$ for different times (0, 24, 48, and 72 h). Each absorbance value represents the average of triplicates of different samples analyzed.

correlates well with the ability to decrease expression of *bcl-2* (30, 31). In the present study, we showed that the expression of *bcl-2* was decreased in a concentration-dependent manner correlated with the percentage of apoptotic NB4 cells treated by TI, suggesting that Bcl-2 may play a role in TI-mediated apoptosis of NB4 cells. Mitochondrial-dependent apoptosis is often through the activation of a proapoptotic factor of the Bcl-2 family. Thus, one possible role of Bcl-2 in the prevention of apoptosis is to block cytochrome *c* release from mitochondria. Indeed, we found the expression of the *bax* proapoptotic gene during TI treatment, and the release of cytochrome *c* was also observed in the present study.

Recently, Bax has been shown to induce cytochrome *c* release and caspase activation in vivo (19) and in vitro (20). This release was reportedly dependent upon induction of the mitochondrial permeability transition, an event that is associated with disruption of the mitochondrial inner transmembrane potential ($\Delta\psi$) (21) and has been implicated in a variety of apoptotic phenomena (22). On the basis of these results, it is reasonable to conclude that TI induces apoptosis in promyelocytic leukemia NB4 cells via a Fas (CD95)- and mitochondria-mediated pathway.

Effects of TI on Caspase-3 or -8 Activity. Treatment with TI for 0, 24, 48, and 72 h significantly induced caspase-3 and -8 activities in NB4 cells (Figure 6). NB4 cells were treated with or without 100 $\mu\text{g/mL}$ TI for 72 h (Figure 6). To measure the caspase-3 or -8 activity of the cell extracts directly and quantitatively, Ac-DEVD-pNA and Ac-IETD-pNA, specific colorimetric substrates of caspase-3 and -8 respectively, were used. The results demonstrated that caspase-3 was activated during TI-induced apoptotic process. Actually, TI also activated caspase-8, although not as strongly as it did caspase-3.

Caspases are believed to play a crucial role in mediating various apoptotic responses. A model has been proposed in which two different caspases, caspase-8 and -9, mediated distinct types of apoptotic stimuli (32, 33). The cascade led by caspase-8 is involved in death receptor-mediated apoptosis such as the one triggered by Fas. Ligation of Fas by Fas ligand results in sequential recruitment of Fas-associated death domain (FADD) and procaspase-8 to the death domain of Fas to form the death-inducing signaling complex, leading to cleavage of procaspase-

8, with the consequent generation of active caspase-8. Active caspase-8 then activates downstream effector caspases through cleavage of Bid, committing the cell to apoptosis (34). The mechanisms by which cytotoxic drugs kill leukemic cells are not well understood, although the activation of caspase-3 has been involved in the important signaling pathway of apoptosis (35). However, further investigations will be needed to clarify the role of death receptors such as Fas (CD95) and TNF-receptor by their ligands.

In this paper, we showed that the impairment of mitochondria in TI-treated NB4 cells was followed by caspase-3 activation. In addition, TI treatment led to a time-dependent proteolysis of Bcl-2 in NB4 cells. The change of this antiapoptotic protein could also be prevented by Ac-DEVD-CHO (caspase-3 inhibitor), suggesting that caspase-3 activity must be crucial in this specific proteolytic process during TI-induced cell death. Bcl-2 is an integral intracellular membrane protein that inhibits programmed cell death induced by multiple stimuli and acts upstream of caspases by blocking the release of cytochrome *c* from mitochondria (36). On the other hand, as shown in this paper, Bcl-2 can also serve as a substrate of caspases and, under certain conditions, be cleaved and converted into a Bax-like proapoptotic molecule (37). Taken together, these results suggest that TI-induced apoptosis is associated with the release of cytochrome *c* and the activation of caspase-3 involving Bcl-2 cleavage.

In conclusion, from these results we suggest that TI shows cytotoxicity through the arrest of the cell cycle at the G1 phase and induction of apoptosis. We propose that TI of sweet potato may be useful as a cancer-preventive substance.

LITERATURE CITED

- Hengartner, M. O. The biochemistry of apoptosis. *Nature* **2000**, *407*, 770–776.
- Earnshaw, W. C.; Martins, L. M.; Kaufmann, S. H. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **1999**, *68*, 383–424.
- Nicholson, D. W. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* **1999**, *6*, 1028–1042.
- Suda, T.; Takahashi, T.; Golstein, P.; Nagata, S. Molecular cloning and expression of the Fas ligand, a novel member of the TNF family. *Cell* **1993**, *75*, 1169–1178.
- Medema, J. P.; Scaffidi, C.; Kischkel, F. C.; Shevchenko, A.; Mann, M.; Krammer, P. H.; Peter, M. E. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J.* **1997**, *16*, 2794–2804.
- Scaffidi, C.; Medema, J. P.; Krammer, P. H.; Peter, M. E. FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. *J. Biol. Chem.* **1997**, *272*, 26953–26958.
- Nagata, S. Apoptosis by death receptor. *Cell* **1997**, *88*, 355–365.
- Nicholson, D. W.; Thornberry, N. A. Caspases: killer proteases. *Trends Biochem. Sci.* **1997**, *22*, 299–306.
- Shimizu, S.; Narita, M.; Tsujimoto, Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* **1999**, *399*, 483–487.
- Katdare, M.; Jinno, H.; Osborne, M. P.; Telang, N. T. Negative growth regulation of oncogene-transformed human breast epithelial cells by phytochemicals: role of apoptosis. *Ann. N. Y. Acad. Sci.* **1999**, *889*, 247–252.
- Katdare, M.; Osborne, M. P.; Telang, N. T. Inhibition of aberrant proliferation and induction of apoptosis in pre-neoplastic human mammary epithelial cells by natural phytochemicals. *Oncol. Rep.* **1998**, *5*, 311–315.

- (12) Ryan, C. A. Proteolytic enzymes and their inhibitors in plants. *Annu. Rev. Plant Physiol.* **1973**, *24*, 173–196.
- (13) Ryan, C. A. Protease inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. *BioEssays* **1989**, *10*, 20–24.
- (14) Sohonie, K.; Bhandarker, A. P. Trypsin inhibitors in Indian foodstuffs: I. Inhibitors in vegetables. *J. Sci. Ind. Res.* **1954**, *13B*, 500–503.
- (15) Lin, Y. H.; Chen, H. L. Level and heat stability of trypsin inhibitor activity among sweet potato (*Ipomoea batatas* Lam.) varieties. *Bot. Bull. Acad. Sin.* **1980**, *21*, 1–13.
- (16) Lin, Y. H. Relationship between trypsin-inhibitor activity and water-soluble protein and cumulative rainfall in sweet potato. *J. Am. Soc. Hortic. Sci.* **1989**, *114*, 814–818.
- (17) Hou, W. C.; Lin, Y. H. Polyamine-bound trypsin inhibitors in sweet potato (*Ipomoea batatas* [L.] Lam cv. Tainong 57) storage roots, sprouted roots and sprouts. *Plant Sci.* **1997**, *126*, 11–19.
- (18) Matsuoka, K.; Matsumoto, S.; Hattori, T.; Machida, Y.; Nakamura, K.; Maeshima, M. Vacuolar targeting and posttranslational processing of the precursor to the sweet potato tuberous root storage protein in heterologous plant cells. *J. Biol. Chem.* **1990**, *265*, 19750–19757.
- (19) Lin, Y. H. Trypsin inhibitors of sweet potato: review and prospect. In *Recent Advances in Botany*; Hsing, Y. I., Chou, C. H., Eds.; Academia Sinica Monograph Series 13; Taipei, Taiwan, 1993; pp 179–185.
- (20) Yeh, K. W.; Chen, J. C.; Lin, M. I.; Chen, Y. M.; Lin, C. Y. Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam): a tuber storage protein with trypsin inhibitory activity. *Plant Mol. Biol.* **1997**, *33*, 565–570.
- (21) Hou, W. C.; Huang, D. J.; Lin, Y. H. An aspartic type protease degrades trypsin inhibitors, the major root storage proteins of sweet potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57). *Bot. Bull. Acad. Sin.* **2002**, *43*, 271–276.
- (22) Hou, W. C.; Chen, Y. C.; Chen, H. J.; Liu, Y. H.; Yang, L. L.; Lee, M. H. Antioxidant activities of a 33 kDa root storage protein of sweet potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57). *J. Agric. Food Chem.* **2001**, *49*, 2978–2981.
- (23) Huang, D. J.; Chen, H. J.; Hou, W. C.; Chen, T. E.; Lin, Y. H. Isolation and characterization of cysteine proteinase cDNA from sweet potato (*Ipomoea batatas* Lam ‘Tainong 57’) storage roots. *Plant Sci.* **2005**, *169*, 423–431.
- (24) Huang, D. J.; Chen, H. J.; Hou, W. C.; Chen, T. E.; Lin, Y. H. In vitro reduction of trypsin inhibitor by purified NADPH/thioredoxin system from sprouts of sweet potato (*Ipomoea batatas* (L.) Lam.) storage roots. *Plant Sci.* **2004**, *166*, 435–441.
- (25) Hou, W. C.; Lin, Y. H. Activity staining on polyacrylamide gels of trypsin inhibitors from leaves of sweet potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57) varieties. *Electrophoresis* **1998**, *19*, 212–214.
- (26) Lanotte, M.; Martin-Thouvenin, V.; Najman, S.; Balerini, P.; Valensi, F.; Berger, R. NB4, a maturation inducible cell line with t (15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* **1991**, *77* (5), 1080–1086.
- (27) Higaki, K.; Yano, H.; Kojiro, M. Fas antigen expression and its relationship with apoptosis in human hepatocellular carcinoma and noncancerous tissues. *Am. J. Pathol.* **1996**, *149*, 429–437.
- (28) Nagao, M.; Nakajima, Y.; Hisanaga, M.; Kayagaki, N.; Kanehiro, H.; Aomatsu, Y. The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance in vivo? *Hepatology* **1999**, *30*, 413–421.
- (29) Lamboley, C.; Bringuier, A. F.; Camus, E.; Lardeux, B.; Groyer, A.; Feldmann, G. Overexpression of the mouse Fas gene in human Hep3B hepatoma cells overcomes their resistance to Fas-mediated apoptosis. *Hepatology* **2002**, *36*, 385–394.
- (30) Bhalla, K.; Ibrado, A. M.; Tourkina, E.; Tang, C.; Mahoney, E.; Huang, Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia* **1993**, *57*, 563–568.
- (31) Delia, D.; Aiello, A.; Formelli, F.; Fontanella, E.; Costa, A.; Miyashita, T.; Reed, J. C.; Pierotti, M. A. Regulation of apoptosis induced by retinoid *N*-(4-hydroxyphenyl) retinamide and effect of deregulated Bcl-2. *Blood* **1995**, *85*, 359–367.
- (32) Sun, X. M.; MacFarlane, M.; Zhuang, J.; Wolf, B. B.; Green, D. R.; Cohen, G. M. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.* **1999**, *274*, 5053–5060.
- (33) Zhuang, J.; Cohen, G. M. Release of mitochondrial cytochrome *c* is upstream of caspase activation in chemical-induced apoptosis in human monocytic tumor cells. *Toxicol. Lett.* **1998**, *102*, 121–129.
- (34) Ashkenazi, A.; Dixit, V. M. Death receptors: signaling and modulation. *Science* **1998**, *281*, 1305–1308.
- (35) Datta, R.; Banach, D.; Kojima, H.; Talanian, R. V.; Alnemri, E. S.; Wong, W. W.; Kufe, D. W. Activation of CPP32 protease in apoptosis induced by 1- β -D-arabinofuranosylcytosine and other DNA-damaging agents. *Blood* **1996**, *88*, 1936–1943.
- (36) Adams, J. M.; Cory, S. The Bcl-2 protein family: arbiters of cell survival. *Science* **1998**, *281*, 1322–1326.
- (37) Kirsch, D. G.; Doseff, A.; Chau, B. N.; Lim, D. S.; de Souza-Pinto, N. C.; Hansford, R.; Kastan, M. B.; Lazebnik, Y. A.; Hardwick, J. M. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome *c*. *J. Biol. Chem.* **1999**, *274*, 21155–21161.

Received for review October 19, 2006. Revised manuscript received January 15, 2007. Accepted February 2, 2007.

JF063008M