JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# 6-Shogaol (Alkanone from Ginger) Induces Apoptotic Cell Death of Human Hepatoma p53 Mutant Mahlavu Subline via an Oxidative Stress-Mediated Caspase-Dependent Mechanism

CHUNG-YI CHEN,[†,‡] TSAN-ZON LIU,[‡,§] YI-WEN LIU,[∥] WEI-CHANG TSENG,[⊥] RAY H. LIU,[⊥] FUNG-JOU LU,[#] YU-SHAN LIN,[⊥] SHIH-HSIEN KUO,*[,†] AND CHING-HSEIN CHEN*[,∥]

Basic Medical Science Education Center and Department of Medical Technology, Fooyin University, Kaohsiung County 831, Taiwan, Graduate Institute of Medical Biotechnology, Chang Gung University, Kwei-Shan, Taoyuan 333, Taiwan, Graduate Institute of Biopharmaceutics, College of Life Sciences, National Chiayi University, 300 University Road, Chiayi 600, Taiwan, and Department of Applied Chemistry, Chung Shan Medical University, Taichung 402, Taiwan

Mahlavu cells, poorly differentiated and p53 mutants of a human hepatoma subline, are known to be highly refractory to a number of chemotherapeutic agents and radiotherapy due to their high expressions of multidrug resistance gene-1 (MDR-1) and Bcl-2 proteins. Thus, it is desirable to search for an alternative strategy for effective eradication of this type of cancer cells. We present evidence here for the first time that 6-shogaol (6-SG), an alkanone isolated from the rhizomes of ginger, can effectively induce apoptotic cell death of Mahlavu cells via an oxidative stress-mediated caspase-dependent mechanism. The cascade of events in 6-SG-induced apoptosis of these cells involved an initial overproduction of reactive oxygen species (ROS) followed by a severe depletion of intracellular glutathione (GSH) contents. Both events consequently entailed a significant drop in mitochondrial transmembrane potential ($\Delta\Psi_m$), which ultimately activated the activities of caspases 3/7 resulting in the DNA fragmentation. Interestingly, we also found that *N*-acetylcysteine (NAC), an antioxidant and a precursor of GSH biosynthesis, could offer a near complete protection of apoptotic cell death exerted by 6-SG. Similarly, exogenously added GSH could also provide protection with an equal efficacy. However, it was paradoxical that both Boc-Asp(OMe)-fmk (a broad caspases inhibitor) and cyclosporin A (an mitochondrial permeability transition opening inhibitor) could only partially protect these cells from 6-SG-induced apoptosis. Taking these data into consideration, it is obvious that GSH depletion is the major contributing factor in arbitrating 6-SG-induced apoptosis of Mahlavu cells. In conclusion, we provide here a novel modality that can help to eradicate a p53 mutant of human hepatoma cells by using a natural consistent isolated form of ginger. These data also provide evidence to reaffirm the notion that consumption of certain foodstuffs can be beneficial to health because some of the constituents contained in them may be anticarcinogenic.

**KEYWORDS: 6-Shogaol; apoptosis; caspases 3/7; oxidative stress; GSH depletion; Mahlavu cells**

## INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) is extensively used in cooking and is known for its flavor, pungency, and pleasing perfume (*1*). 6-Shogaol (6-SG) is one of several phenolic alkanones isolated from the rhizomes of ginger (**Figure 1**)

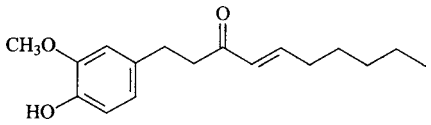



**Figure 1.** Structure of 6-SG.

(*2*−*4*). A number of in vitro and in vivo studies have found that 6-SG possesses many medical and biological effects including antiemetic, vasodilating, antibacterial, antifouling, antitussive, and antihepatotoxic properties (*5*−*10*). In addition, 6-SG, a guaiacol-containing compound, has also been demonstrated to possess antioxidative properties (*11*). However, despite

* To whom correspondence should be addressed. Tel: 886-5-2717837. Fax: 886-5-2717778. E-mail: chench@mail.ncyu.edu.tw (C.-H.C.) or ns215@mail.fy.edu.tx (S.-H.K.).
† Basic Medical Science Education Center, Fooyin University.
‡ Both authors contributed equally to this work.
§ Chang Gung University.
∥ National Chiayi University.
⊥ Department of Medical Technology, Fooyin University.
# Chung Shan Medical University.

10.1021/jf0624594 CCC: $37.00 © 2007 American Chemical Society
Published on Web 01/05/2007

numerous biological functions of 6-SG that have been identified, reports dealing with its anticancer effects have been scanty.

It has been documented that a variety of constituents isolated from foods and herbal plants are sometimes proved to be anticarcinogenic and antimutagenic. Ironically, all of these constituents show strong antioxidative capacities. For example, crude extracts of *Toona sinensis* were reported to possess anticancer and hypoglycemic effects (*12*). Resveratrol, a constituent isolated from seeds of grapes, was demonstrated to possess anticarcinogenic activity because of its capability to serve as an antioxidant, antiinflammatory agent as well as a phase II enzyme inducer (*13*). On the basis of these arguments, it can be inferred that 6-SG, a structural analogue to resveratrol, may also exhibit anticarcinogenic activity.

The specific aim of the present study is therefore to attest whether or not 6-SG can exert a carcinogenic effect. Here, we present evidence that 6-SG can indeed provoke apoptotic cell death on human hepatoma p53 mutant Mahlavu sublines via a mechanism involving an oxidative stress-mediated caspase-dependent pathway.

## MATERIALS AND METHODS

**Plant Material.** The roots of *Z. officinale* (Ginger) were purchased from a local market of Kaohsiung in Taiwan in July 2005. A voucher specimen was deposited at Fooyin University.

**Extraction and Isolation.** The roots (4.1 kg) of *Z. officinale* were chipped and air-dried and extracted repeatedly with $CHCl_3$ at room temperature. The combined $CHCl_3$ extracts were then evaporated further separated into 14 fractions by column chromatography on silica gel with gradients of *n*-hexane/$CHCl_3$. Fraction 11 eluted with $CHCl_3$−MeOH (40:1) was next repeatedly subjected to silica gel CC and yielded 6-SG (70 mg). As a result, 6-SG (purity >99%) was isolated. The structure of 6-SG was identified by spectroscopic analysis (**Figure 1**).

**Cell Line and Reagents.** The Mahlavu cell line was obtained from the American Type Culture Collection (Rockville, MD). The "Apo-BrdU" kits were purchased from BD Pharmingen (San Diego, CA). The Apo-one homogeneous caspase-3/7 assay kit was obtained from Promega (Madison, WI). The chloromethylflourescein diacetate (CMF-DA) employed herein was obtained from Invitrogen Taiwan, Ltd. *N*-Acetylcysteine (NAC), cyclosporin A, propidium iodide (PI), rhodamine 123, 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA), Boc-Asp(OMe)-fmk, NAC, glutathione (GSH), Dulbecco's modified Eagle's medium (DMEM), and other chemicals were bought from Sigma Chemical Co. (St. Louis, MO).

**Cell Cultures and Treatment.** The basal medium for Mahlavu cell line culture was DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 $\mu$g/mL streptomycin. The stock solution of 6-SG (100 mM) was liquefied in DMSO, and various concentrations were prepared in the previous basal medium with a final DMSO concentration of 0.1%.

**Cell Cycle Analysis of Cell Line.** Various cells were individually cultured in 60 mm tissue culture dishes. The culture medium was replaced by a new medium when the cells were 80% growth confluent, and then, it was treated with various concentrations of 6-SG for 9 h. After treatment, adherent and floating cells were collected, washed with phosphate-buffered saline (PBS), then fixed with PBS−methanol (1:2, volume/volume) solution, and stored at 4 °C for at least 18 h. Following two more washes with PBS, the cell pellets were stained with the fluorescent probe solution containing PBS, 40 $\mu$g/mL propidium iodide, and 40 $\mu$g/mL DNase-free RNaseA for 30 min at room temperature in the dark. DNA fluorescence of PI-stained cells was evaluated by excitation at 488 nm and detected through a 630/22 nm band-pass filter using a Becton-Dickinson FACS-Calibur flow cytometer. A minimum of 10000 cells were analyzed per sample, and the DNA histograms were gated and analyzed further using Modfit software on a Mac workstation to estimate the percentage of cells in various phases of the cell cycle.

**Measurement of Intracellular Reactive Oxygen Species (ROS).** The production of intracellular ROS was detected by flow cytometry using DCFH-DA (*14*). Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 and 100 $\mu$M 6-SG for 1 and 3 h. After treatment, cells were treated with 10 $\mu$M DCFH-DA for 30 min in the dark, washed once with PBS, detached by trypsinization, collected by centrifugation, and resuspended in PBS. The intracellular ROS, as exhibited by the fluorescence of dichlorofluorescein (DCF), was measured with a Becton-Dickinson FACS-Calibur flow cytometer.

**Measurement of the Cellular Membrane Integrity.** The cellular membrane integrity was detected by propidium iodide staining (*15*). Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then exposed to 0 and 100 $\mu$M 6-SG for 3, 6, and 9 h. After treatment, trypsinized cells were resuspended in PBS and stained for 10 min with 5 $\mu$g/mL propidium iodide. Stained cells were excited by exposure to argon laser at 488 nm, collection of fluorescence emission at 580 nm, and at least 10000 cells counted with a FACS-Calibur flow cytometer, using CellQuest software.

**Measurement of Intracellular GSH Depletion.** The intracellular GSH depletion was determined by flow cytometry after staining with CMF-DA (*16*). Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 and 100 $\mu$M 6-SG for 3, 6, and 9 h. After treatment, adherent and floating cells were collected, washed with PBS, and then resuspended with PBS. It was added at 25 $\mu$M CMF-DA in cell suspensions adjusted at $1-2 \times 10^6$ cells per mL. After 30 min of incubation at 37 °C, cells were washed twice in PBS, resuspended at a concentration of $10^6$ cells/mL in PBS, and analyzed on a Becton-Dickinson FACS-Calibur flow cytometer. The fluorescent dye−thioether adduct was excited at 488 nm, and the fluorescence was collected with a 525 nm band-pass filter. Analyses were performed on at least 10000 cells, and fluorescence intensities were measured on a logarithmic scale of fluorescence of four decades of log. The data were collected, stored, and analyzed with the CellQuest software.

**Measurement of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$).** Rhodamine123 is a fluorescent dye that is incorporated into mitochondria in a $\Delta\Psi_m$-dependent manner (*14*). Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 and 100 $\mu$M 6-SG for 3, 6, and 9 h. After treatment, the culture medium was replaced with a new medium with 5 $\mu$M rhodamine123 for 30 min in the dark. Subsequent to the incubation step, cells were harvested by trypsinization, following which, $\Delta\Psi_m$, as indicated by the fluorescence level of rhodamine123, was analyzed using a Becton-Dickinson FACS-Calibur flow cytometer.

**Measurement of Caspase 3/7 Activities.** Caspase 3/7 activities were evaluated by a method followed from our previous publication with Apo-one homogeneous caspase-3/7 assay kit (*14*). The caspase 3/7 substrate, Z-DEVD-R110, was diluted with a buffer to make the desired amount of homogeneous substrate reagent. Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with a new medium when the cells were 80% confluent and then treated with 100 $\mu$M 6-SG for 1, 3, 6, and 9 h. After treatment, the cells were washed once with PBS, detached by trypsinization, and collected by centrifugation. Aliquot $1 \times 10^6$ cells were suspended in a DMEM medium, and then, homogeneous substrate reagents were added to the cells, maintaining a 1:1 ratio of reagent to cell solution. After 1 h of incubation at 37 °C, the cells were washed once with PBS, collected by centrifugation, and suspended in PBS. Substrate cleavage to release free R110 or FITC fluorescence intensity was measured in a Becton-Dickinson FACS-Calibur flow cytometer with excitation wavelength set at 488 nm and emission wavelength at 520 nm.

**TUNEL Assay for Apoptosis.** Following incubation of drugs, TUNEL was followed from our previous publication with the "Apo-BrdU" kits (*14*). Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent. The cells were pretreated with NAC, GSH,

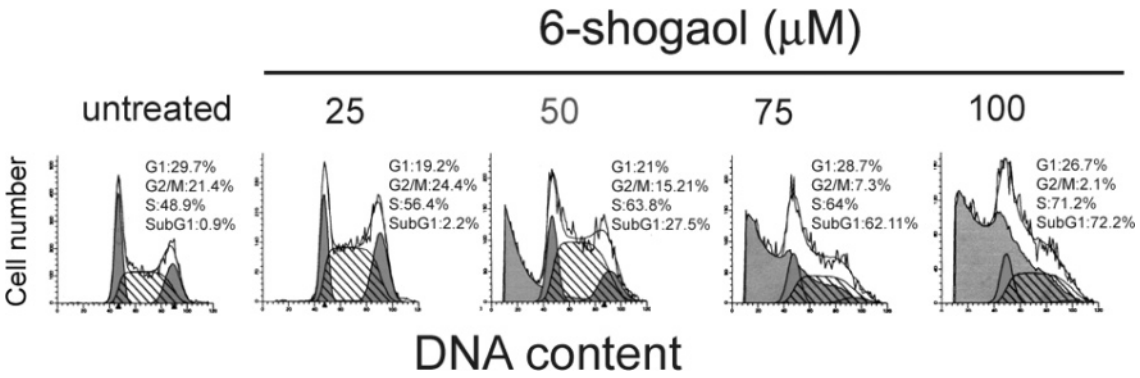



**Figure 2.** Cell cycle and DNA damage analysis in 6-SG-treated Mahlavu cell line. Cells were plated in 60 mm cultured dishes at 80% confluence and then treated with 0, 25, 50, 75, and 100 $\mu$M 6-SG for 9 h. After treatment, adherent and floating cells were harvested, fixed in PBS–methanol (1:2, volume/volume) solution, and stained with PI, followed by flow cytometric analysis. These experiments were performed at least three times, and a representative experiment is presented.

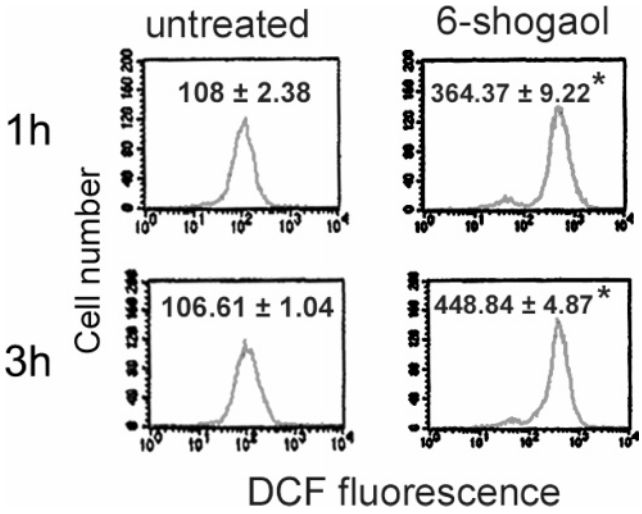



**Figure 3.** Intracellular ROS analysis in 6-SG-treated Mahlavu cells. Cells were plated in 60 mm cultured dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 (untreated) and 100 $\mu$M 6-SG for 1 and 3 h. After drug treatments, cells were treated with 10 $\mu$M DCFH-DA for 30 min in the dark, washed once with PBS, detached by trypsinization, collected by centrifugation, and suspended in PBS. The intracellular ROS, as indicated by the fluorescence of dichlorofluorescein (DCF), was measured with a Becton-Dickinson FACS-Calibur flow cytometer. The data in each panel represent the DCF fluorescence intensity within the cells. The values shown are means ± standard errors ($n$ = 6 of individual experiments). Significant differences from the untreated group are $P < 0.05$ (*).

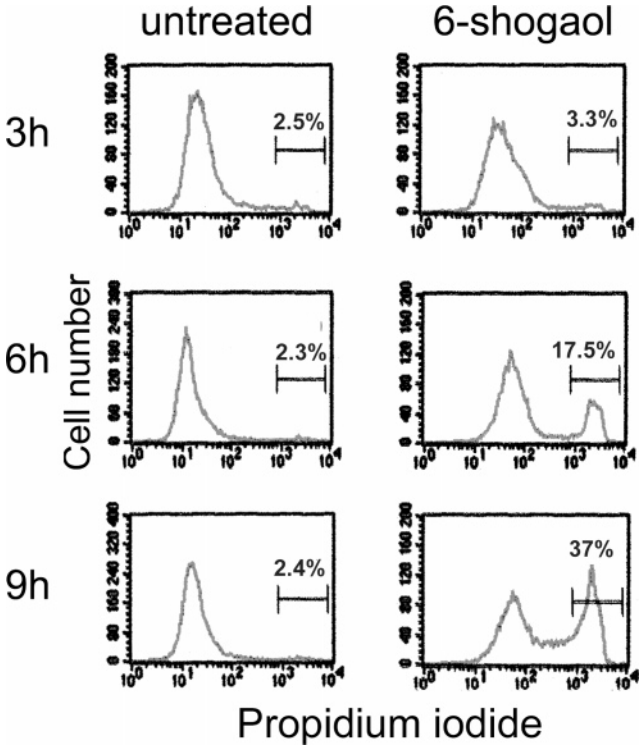



**Figure 4.** Cell membrane damage anaylsis in 6-SG-treated Mahlavu cells. Cells were plated in 60 mm cultured dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 (untreated) and 100 $\mu$M 6-SG for 3, 6, and 9 h. After treatment, trypsinized cells were resuspended in PBS and stained for 10 min with 5 $\mu$g/mL propidium iodide and then measured by flow cytometry. Data represent the percentage of cells displaying cell membrane damaged cells. These experiments were performed at least three times, and a representative experiment is presented.

Boc-Asp(OMe)-fmk, and cyclosporine A for 1 h and then treated with 100 $\mu$M 6-SG for 9 h. After drug treatments, both floating and adherent cells were harvested and fixed using 1% paraformaldehyde for 30 min at 4 °C. After fixation, cells were permeated with 70% ethanol for 30 min at 4 °C for at least 18 h, followed by two washes with PBS containing 0.2% BSA. To label DNA strand breaks, $1-2 \times 10^6$ cells were incubated with 50 $\mu$L of TUNEL reaction buffer containing terminal deoxynucleotidyl transferase and fluorescein-BrdUTP, and it was then incubated for 1 h at 37 °C in a humidified 5% $CO_2$-in-air atmosphere. Cells were washed twice with PBS, suspended in PBS containing 50 $\mu$g/mL of PI and 50 $\mu$g/mL of DNase-free RNase A for 30 min at room temperature in the dark, and then analyzed by a Becton-Dickinson FACS-Calibur flow cytometer.

**Statistical Analysis.** Data were presented as means ± standard errors from at least three independent experiments and analyzed using Student's $t$-test. A $P$ value of less than 0.05 was considered as statistically significant.

## RESULTS

**6-SG-Induced DNA Damages of Mahlavu Cells.** We first tested the DNA damage at concentrations ranging from 25 to 100 $\mu$M 6-SG in Mahlavu cells by flowcytometry and PI staining. As shown in **Figure 2**, the SubG1 fraction of cells treated with 25 $\mu$M 6-SG was merely 2.2% that was comparable to the untreated control (0.9%). These data indicated that the cells treated with this concentration of 6-SG caused minimal DNA damage. In contrast, a drastic increment of the obtainment of SubG1 fractions occurred when the concentrations of 6-SG treatment were beyond 50 $\mu$M. For the subsequent experiments,

we therefore selected a concentration of 100 $\mu$M for the treatment of Mahlavu cells because extensive DNA damage could be vividly observed by this concentration used (72.2%).

**6-SG-Induced Increased ROS Production of Mahlavu Cells.** We evaluated the effect of 6-SG on the production of intracellular of ROS by the DCFH-DA staining technique. As shown in **Figure 3**, Mahlavu cells treated with 100 $\mu$M 6-SG caused approximately 4-fold increases in DCF fluorescence intensity undergoing 3 h of treatment when compared to the untreated counterparts.

**6-SG-Damaged Cell Membrane Integrity of Mahlavu Cells.** We further studied if 6-SG could damage the cell membranes of Mahlavu cells by using PI staining technique. The principle of this technique is that when the cell membrane is damaged, PI can cross the cell membrane to the nucleus. Conversely, if the membrane integrity is intact, PI across the membrane can be prevented. As indicated in **Figure 4**, when the cells were treated with 100 $\mu$M 6-SG, the PI-positive cells were drastically increased to 17.5 and 37.0%, respectively, after 6 and 9 h of treatment. In comparison, the untreated controls merely generated <3% of PI-positive cells.

**6-SG-Caused GSH Depletion of Mahlavu Cells.** We studied the possibility that 6-SG could also deplete intracellular GSH contents by using flowcyotmetric analysis based on CMF-DA staining technique. CMF-DA, a nonfluorescence compound, can enter the intracellular compartment and react with esterase to allow CMF trapped inside the cell. The interaction of CMF with thiol group of GSH will result in the generation of fluorescence. Thus, the decrease in fluorescence reflects the depletion of the availability of GSH. As shown in **Figure 5**, 6-SG treatment resulted in drastic decreases in CMF fluorescence in a time-dependent fashion. It is interesting to note that approximately 93% of GSH-depleted cells can be observed after 24 h of treatment with 100 $\mu$M 6-SG.

**6-SG-Induced $\Delta\Psi_m$ Decrease of Mahlavu Cells.** Several investigations have demonstrated that apoptosis requires a process that concomitantly proceeds with an irreversible loss of mitochondrial membrane potential ($\Delta\Psi_m$) (*17*, *18*). We thus proceeded to examine the possibility that 6-SG could induce apoptosis via the mitochondrial signaling pathway. In this study, the $\Delta\Psi_m$ was evaluated by using the rhodamine 123 fluorescent dye, which specifically accumulates within the mitochondrial compartment in a $\Delta\Psi_m$-dependent manner. Reduced $\Delta\Psi_m$ is reflected by reduced staining with rhodamine 123. As shown in **Figure 6**, the untreated cells maintained rhodamine 123 fluorescence readings at 505−512. The rhodamine 123 fluorescence was significantly decreased to 414.15 ± 13.95, 156.52 ± 11.82, and 145.86 ± 1.16 after treatment of 100 $\mu$M 6-SG at 3, 6, and 9 h, respectively.

**6-SG-Induced Activation of Caspase 3/7 in Mahlavu Cells.** Caspases, a group of cysteine proteases, have been demonstrated to play a pivotal role in the induction of apoptosis (*19*). Thus, we set out to explore if 6-SG-induced apoptosis of Mahlavu cells proceeded by a caspase-dependent mechanism. As shown in **Figure 7**, when the cells were treated with 100 $\mu$M 6-SG, caspase 3/7 activities rose approximately 3-, 8-, and 9-fold at 1, 3, and 6 h of treatment as compared to untreated controls.

**Effects of Various Affectors in 6-SG-Induced Apoptosis.** We test the effects of NAC (an antioxidant and a precursor for GSH biosynthesis) (*20*), GSH, Boc-Asp(OMe)-fmk (a broad caspases inhibitor) (*19*), and cyclosporin A (an mitochondrial permeability transition-opening inhibitor) (*21*) on 6-SG-induced apoptosis of Mahlavu cells. The cells were pretreated with all of the above-mentioned substances for 1 h, followed by

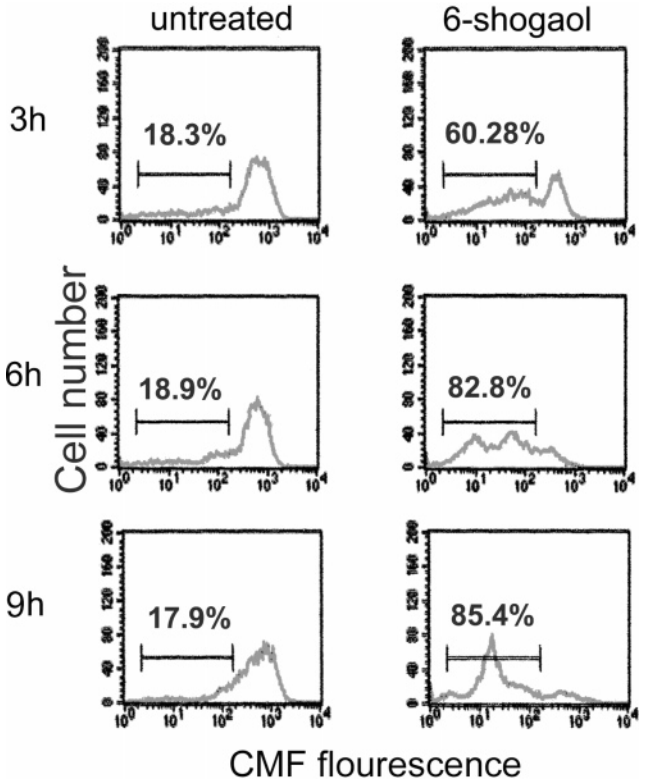



**Figure 5.** GSH depletion anaylsis in 6-SG-treated Mahlavu cells. Cells were plated in 60 mm cultured dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 (untreated) and 100 $\mu$M 6-SG for 3, 6, and 9 h. After treatment, the cells were incubated with 25 $\mu$M CMF-DA for 30 min in 37 °C $CO_2$ incubator and then measured by flow cytometry. Data represent the percentage of cells displaying intracellular GSH negative cells. These experiments were performed at least three times, and a representative experiment is presented.

treatment of the cells with 100 $\mu$M 6-SG. At a designated period of time after these treatments, the cells were analyzed by TUNEL assay. As shown in **Figure 8**, the proportion of apoptotic cells in the untreated group remained remarkably low (0.6%). However, after the cells were treated with 100 $\mu$M 6-SG, the proportion of TUNEL-positive cells rose to 89.3% at 9 h. Interestingly, after pretreatment of the cells with 10 mM NAC or 10 mM GSH, followed by treatment with 100 $\mu$M 6-SG, the proportion of TUNEL-positive cells dropped to a remarkably lower level (0.6 and 1.4%, respectively). These data indicate that NAC and GSH are potent inhibitors of 6-SG-induced apoptotic cell death. In addition, Boc-Asp(OMe)-fmk and cyclosporin A could only offer partial protection against 6-SG-induced apoptotic cell death.

## DISCUSSION

First, the selection of p53 mutants of human hepatoma Mahlavu cells as our experimental model needs some qualifications. The most frequent alteration in human malignancies is deletion or mutation of the p53 gene. p53 is a nuclear phosphoprotein and is a tumor suppressor whose inactivation is strongly correlated with human cancer (*22*, *23*). Wild-type p53 induces the transcription of a number of downstream genes linked to cell cycle arrest (*24*). However, wild-type p53 also represses the transcription of some genes that correlate with the ability of p53 to induce apoptosis (*25*). Loss of p53 repression functions results in decreased susceptibility to apoptotic stimuli.

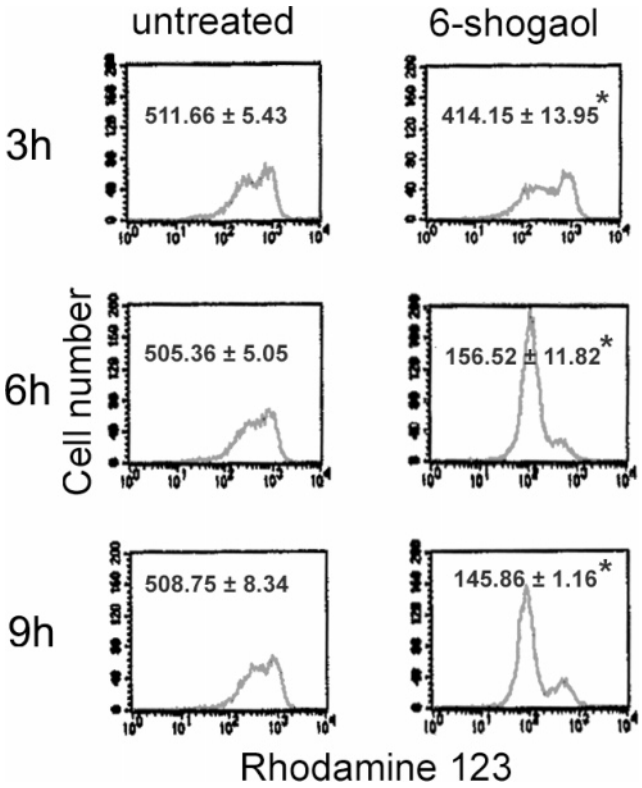



**Figure 6.** Mitochondrial transmembrane potential ($\Delta\Psi_m$) anaylsis in 6-SG-treated Mahlavu cells. Cells were plated in 60 mm cultured dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 (untreated) and 100 $\mu$M 6-SG for 3, 6, and 9 h. After treatment, the cells were incubated with 5 $\mu$M rhodamine 123 for 30 min in 37 °C $CO_2$ incubator and then measured by flow cytometry. Data represent the rhodamine 123 fluorescence intensity within the cells. Values shown are means ± standard deviations ($n = 6$). Significant differences from the untreated group are $P < 0.05$ (*).

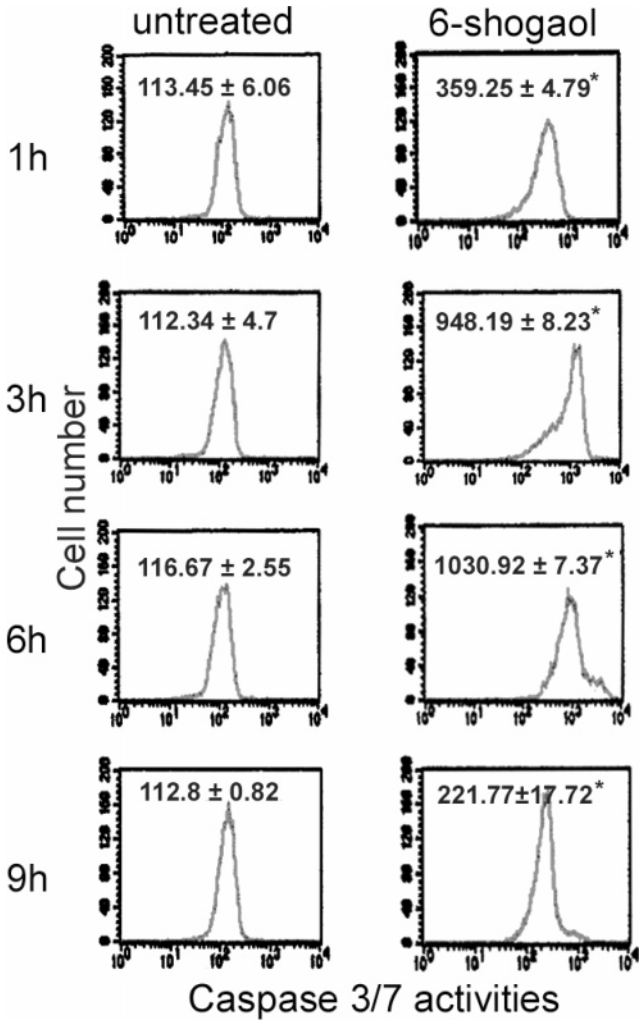



**Figure 7.** Caspase 3/7 activities anaylsis in 6-SG-treated Mahlavu cells. Cells were plated in 60 mm cultured dishes. The culture medium was replaced with new medium when the cells were 80% confluent and then treated with 0 (untreated) and 100 $\mu$M 6-SG for 1, 3, 6, and 9 h. After treatment, the Z-DEVD-R110 substrate was added to the cells for 1 h at 37 °C. Data represent the intracellular caspase 3/7 activities within the cells. Values shown are means ± standard deviations ($n = 6$). Significant differences from the untreated group are $P < 0.05$ (*).

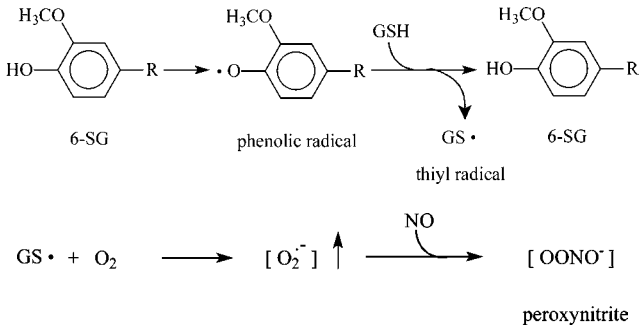
Indeed, mutations in p53 correlate with chemotherapeutic resistance (*26*). Mahlavu cells, being a p53 mutant, have been demonstrated by us to be resistant to some chemotherapeutic agents and acquired radioresistance due to high expression of Bcl-2 protein (Liu et al., unpublished data). Therefore, it is of necessity to find an alternative approach and/or agent that can eradicate these cells effectively.

The present study demonstrated that 6-SG, at a proper concentrations (>50 $\mu$M), could provoke DNA damage and apoptosis in Mahlavu cells. The cascade of events associated with this apoptotic process involved an initial increased production of ROS and GSH depletion. This ROS/GSH depletion-evoked oxidative stress subsequently led to the changes in mitochondrial transmembrane potential and ultimately resulted in the activation of caspase 3/7 activities. Interestingly, our data also revealed that 6-SG-provoked ROS production preceded the depletion of GSH contents.

There are two pathways that may help to interpret the possible mechanism(s) associated with the 6-SG-induced GSH depletion in Mahlavu cells. First, we have previously demonstrated that Mahlavu cells could express increased amounts of nitric oxide (NO) constitutively due to its high contents of inducible NO synthase (iNOS) (data not shown). The depletion of intracellular GSH can then proceed via the interaction of NO and GSH:

$$NO + GSH \rightarrow GSNO + H^+ \quad (1)$$

Alternatively, 6-SG, being a guaiacol-containing compound, is able to form a phenolic radical via reaction with an oxidant. The resulting phenolic radicals are capable of acquiring a hydrogen atom to return to its original configuration with commensurate formation of thiyl radical (GS•). The latter can thus generate superoxide radical ($O_2^{\bullet -}$) in the presence of molecular oxygen. Subsequently, the interaction between $O_2^{\bullet -}$ and NO will generate peroxynitrite ($OONO^-$). The latter could be the culprit responsible for the induction of apoptotic cell death. The possible sequences of these reactions are depicted below:



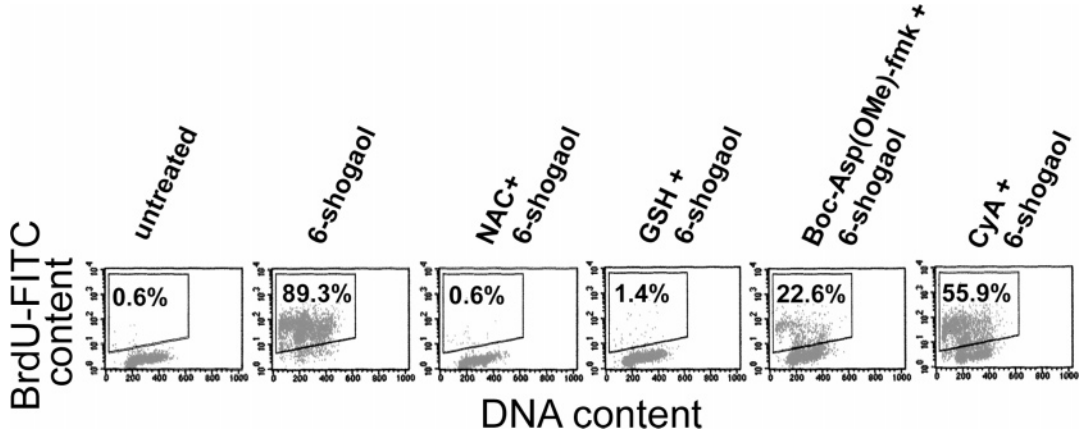
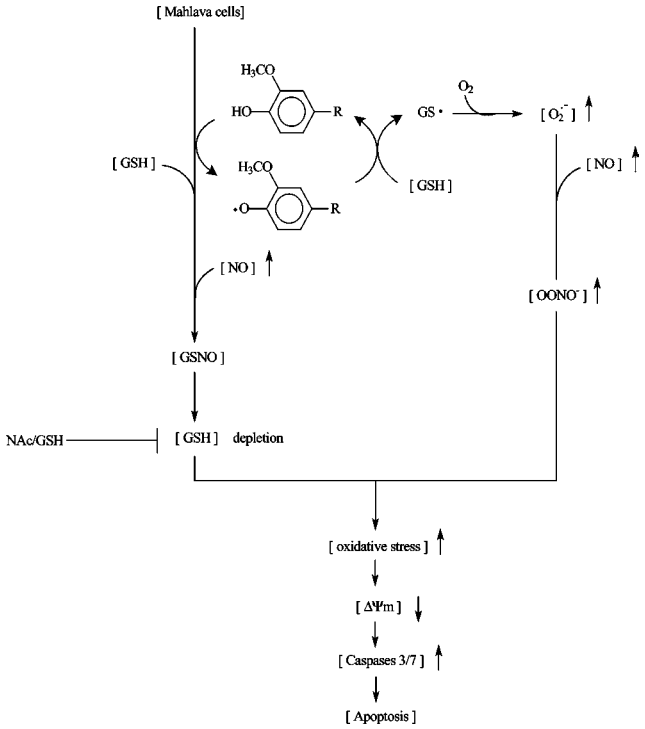



**Figure 8.** Evaluation of critical role in 6-SG-induced apoptosis. Mahlavu cells were cultured in 60 mm tissue culture dishes. The culture medium was replaced with a new medium when the cells were 80% confluent. Mahlavu cells were treated with 0 (untreated) or 100 $\mu$M 6-SG alone for 9 h, pretreated with 10 mM NAC, 10 mM GSH, 100 $\mu$M Boc-Asp(OMe)-fmk, or 5 $\mu$M cyclosporin A (CyA) for 1 h, followed by 100 $\mu$M 6-SG treatment for 9 h, and then proceeded to the TUNEL assay. The representative plots depict DNA content on the *x*-axis and BrdU-FITC-labeled apoptotic DNA strand breaks on the *y*-axis. Data represent the percentage of cells in the upper box of apoptosis. These experiments were performed at least three times, and a representative experiment is presented.

Several reports indicated that intracellular GSH depletion could result in mitochondria dysfunction (*27*, *28*). In neurons, treatment with monochlorobimane, a GSH-depleting agent, induced mitochondrial dysfunction, with transmembrane potential breakdown followed by cytoplasmic $Ca^{2+}$ deregulation. Furthermore, in some lung cancer cell lines, GSH depletion had been found to enhance Bax protein translocation to mitochondrial membrane and then sensitized cells to apoptosis (*29*). In our 6-SG-treated Mahlavu cells, severe depletion of intracellular GSH contents (>80%) could be detected at 6−9 h after 6-SG treatment, which coincidently correlated with the severe drops in mitochondrial transmembrane potential ($\Delta\Psi_m$). Thus, intracellular GSH depletion evoked by 6-SG may be one of key elements in arbitrating apoptosis.

Alternatively, increased NO production by Mahlavu cells can be very disruptive in destroying the mitochondrial function, a procedure promoted by its high reactivity with $O_2^{\bullet-}$. This interaction increases the $OONO^-$ production, further provoking mitochondrial dysfunction and damage to cells by apoptosis (*30*). Our data are also consistent with this notion. On the basis of this argument, we speculate that $OONO^-$ formation may also contribute at least in part to the induction of apoptotic cell death provoked by 6-SG.

The ultimate step of apoptosis is proceeded by the activation of caspase activity. In our studies, we found that ROS production and intracellular GSH depletion coexisted in 6-SG-treated cells at 3 h. This was followed by a rapid increment of caspases 3/7 activities. Also, a near complete suppression of 6-SG-induced apoptosis by NAC and GSH further strengthens the notion that GSH depletion is the major contributing factor involved in the arbitration of apoptosis. Taking all data into consideration, a possible cascade of events of 6-SG-provoked apoptosis can be postulated below:




## ABBREVIATIONS USED

6-SG, 6-shogaol; ROS, reactive oxygen species; CMF-DA, chloromethylfluorescein diacetate; PI, prodidium iodide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; NAC, *N*-acetylcysteine; iNOS, inducible nitric oxide synthase; $O_2^-$, superoxide anion; NO, nitric oxide; $OONO^-$, peroxynitrite; $GS^{\bullet}$, thiyl radical.

## LITERATURE CITED

(1) Agarwal, M.; Walia, S.; Dhingra, S.; Khambay, B. P. Insect growth inhibition, antifeedant and antifungal activity of compounds isolated/derived from *Zingiber officinale* Roscoe (ginger) rhizomes. *Pest. Manage. Sci.* **2001**, *57*, 289−300.

(2) Wu, T. S.; Wu, Y. C.; Wu, P. L.; Chern, C. Y.; Leu, Y. L.; Chan, Y. Y. Structure and synthesis of (*n*)-dehydroshogaols from *Zingiber officinale*. *Phytochemistry* **1998**, *48*, 889−891.

(3) Kikuzaki, H.; Tsai, S. M.; Nakatani, N. Gingerdiol related compounds from the rhizomes of *Zingiber officinale*. *Phytochemistry* **1992**, *31*, 1783−1786.

(4) Kikuzaki, H.; Nakatani, N. Cyclic diarylheptanoids frrom rhizomes of *Zingiber officinale*. *Phytochemistry* **1996**, *43*, 273−277.

(5) Abdel-Aziz, H.; Windeck, T.; Ploch, M.; Verspohl, E. J. Mode of action of gingerols and shogaols on 5-HT3 receptors: Binding studies, cation uptake by the receptor channel and contraction of isolated guinea-pig ileum. *Eur. J. Pharmacol.* **2006**, *530*, 136−143.

(6) Ghayur, M. N.; Gilani, A. H.; Afridi, M. B.; Houghton, P. J. Cardiovascular effects of ginger aqueous extract and its phenolic constituents are mediated through multiple pathways. *Vasc. Pharmacol.* **2005**, *43*, 234−241.
(7) Mahady, G. B.; Pendland, S. L.; Yun, G. S.; Lu, Z. Z.; Stoia, A. Ginger (*Zingiber officinale* Roscoe) and the gingerols inhibit the growth of Cag A+ strains of Helicobacter pylori. *Anticancer Res.* **2003**, *23*, 3699−3702.
(8) Etoh, H.; Kondoh, T.; Noda, R.; Singh, I. P.; Sekiwa, Y.; Morimitsu, K.; Kubota, K. Shogaols from *Zingiber officinale* as promising antifouling agents. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 1748−1750.
(9) Hikino, H.; Kiso, Y.; Kato, N.; Hamada, Y.; Shioiri, T.; Aiyama, R.; Itokawa, H.; Kiuchi, F.; Sankawa, U. Antihepatotoxic actions of gingerols and diarylheptanoids. *J. Ethnopharmacol.* **1985**, *14*, 31−39.
(10) Suekawa, M.; Ishige, A.; Yuasa, K.; Aburada, M.; Hosoya, E. Pharmacological studies on ginger. I. Pharmacological actions of pungent constitutents, (6)-gingerol and (6)-shogaol. *J. Pharmacobiodyn.* **1984**, *7*, 836−848.
(11) Katiyar, S. K.; Agarwal, R.; Mukhtar, H. Inhibition of tumor promotion in sencar mouse skin by ethanol extract of *Zingiber officinale* rhizome. *Cancer Res.* **1996**, *56*, 1023−1030.
(12) Chang, H. C.; Hung, W. C.; Huang, M. S.; Hsu, H. K. Extract from leaves of *Toona Sinensis* roemor exerts potent antiproliferative effect on human lung cancer cells. *Am. J. Chin. Med.* **2002**, *30*, 307−314.
(13) Halliwell, B.; Gutteridge, J. M. C. Nutrition health and oxidative stress. *Free Radicals in Biology and Medicine*, 3rd ed.; Oxford University Press: London, England, 1998; pp 810.
(14) Hsieh, T. J.; Liu, T. Z.; Lu, F. J.; Hsieh, P. Y.; Chen, C. H. Actinodaphnine induces apoptosis through increased nitric oxide, reactive oxygen species and down-regulation of NF-$\kappa$B signaling in human hepatoma Mahlavu cells. *Food Chem. Toxicol.* **2006**, *44*, 344−354.
(15) Chen, C. H.; Hsieh, T. J.; Liu, T. Z.; Chern, C. L.; Hsieh, P. Y.; Chen, C. Y. Annoglabayin, a novel dimeric kaurane diterpenoid, and apoptosis in Hep G2 cells of annomontacin from the fruits of *Annona glabra*. *J. Nat. Prod.* **2004**, *67*, 1942−1946.
(16) Hsieh, T. J.; Liu, T. Z.; Chia, Y. C.; Chern, C. L.; Lu, F. J.; Chuang, M. C.; Mau, S. Y.; Chen, S. H.; Syu, Y. H.; Chen, C. H. Protective effect of methyl gallate from *Toona sinensis* (Meliaceae) against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells. *Food Chem. Toxicol.* **2004**, *42*, 843−850.
(17) Liu, G. Y.; Hung, Y. C.; Hsu, P. C.; Liao, Y. F.; Chang, W. H.; Tsay, G. J.; Hung, H. C. Ornithine decarboxylase prevents tumor necrosis factor alpha-induced apoptosis by decreasing intracellular reactive oxygen species. *Apoptosis* **2005**, *10*, 569−581.
(18) Wang, Y.; Perchellet, E. M.; Ward, M. M.; Lou, K.; Zhao, H.; Battina, S. K.; Wiredu, B.; Hua, D. H.; Perchellet, J. P. Antitumor triptycene analogs induce a rapid collapse of mitochondrial transmembrane potential in HL-60 cells and isolated mitochondria. *Int. J. Oncol.* **2006**, *28*, 161−172.
(19) Riedl, S. J.; Shi, Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 897−907.
(20) Dekhuijzen, P. N. Antioxidant properties of *N*-acetylcysteine: Their relevance in relation to chronic obstructive pulmonary disease. *Eur. Respir. J.* **2004**, *23*, 629−636.
(21) Whiteman, M.; Rose, P.; Siau, J. L.; Cheung, N. S.; Halliwell, B.; Armstrong, J. S. Hypochlorous acid-mediated mitochondrial dysfunction and apoptosis in human hepatoma HepG2 and human fetal liver cells: Role of mitochondrial permeability transition. *Free Radical Biol. Med.* **2005**, *38*, 1571−1584.
(22) Hollstein, M.; Sidransky, D.; Vogelstein, B.; Harris, C. C. p53 mutations in human cancers. *Science* **1991**, *253*, 49−53.
(23) Levine, A. J.; Momand, J.; Finlay, C. A. The p53 tumour suppressor gene. *Nature* **1991**, *351*, 453−456.
(24) Subler, M. A.; Martin, D. W.; Deb, S. Inhibition of viral and cellular promoters by human wild-type p53. *J. Virol.* **1992**, *66*, 4757−4762.
(25) Sabbatini, P.; Chiou, S. K.; Rao, L.; White, E. Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein. *Mol. Cell. Biol.* **1995**, *15*, 1060−1070.
(26) Perego, P.; Giarola, M.; Righetti, S. C.; Supino, R.; Caserini, C.; Delia, D.; Pierotti, M. A.; Miyashita, T.; Reed, J. C.; Zunino, F. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res.* **1996**, *56*, 556−562.
(27) Friesen, C.; Kiess, Y.; Debatin, K. M. A critical role of glutathione in determining apoptosis sensitivity and resistance in leukemia cells. *Cell Death Differ.* **2004**, Suppl. 1, S73−S85.
(28) Vesce, S.; Jekabsons, M. B.; Johnson-Cadwell, L. I.; Nicholls, D. G. Acute glutathione depletion restricts mitochondrial ATP export in cerebellar granule neurons. *J. Biol. Chem.* **2005**, *280*, 38720−38728.
(29) Honda, T.; Coppola, S.; Ghibelli, L.; Cho, S. H.; Kagawa, S.; Spurgers, K. B.; Brisbay, S. M.; Roth, J. A.; Meyn, R. E.; Fang, B.; McDonnell, T. J. GSH depletion enhances adenoviral bax-induced apoptosis in lung cancer cells. *Cancer Gene Ther.* **2004**, *11*, 249−255.
(30) Heck, D. E.; Kagan, V. E.; Shvedova, A. A.; Laskin, J. D. An epigrammatic (abridged) recounting of the myriad tales of astonishing deeds and dire consequences pertaining to nitric oxide and reactive oxygen species in mitochondria with an ancillary missive concerning the origins of apoptosis. *Toxicology* **2005**, *208*, 259−271.

**Received for review August 28, 2006. Revised manuscript received November 27, 2006. Accepted December 5, 2006. This work was supported by two grants from the National Science Council of ROC [NSC 95-2320-B-415-005 (C.-H.C.)] and [NSC-94-2320-B-242-009(C.-Y.C.)] and a grant from Chung Gung Medical Research Fund (CMRPD140041) (T.-Z.L.).**

JF0624594