

Goniothalamine induces cell cycle-specific apoptosis by modulating the redox status in MDA-MB-231 cells

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Abstract

Goniothalamine, a natural occurring styryl-lactone, is a novel compound with putative anticancer activities. In the present study, the mechanism of action of goniothalamine was further investigated in human breast cancer MDA-MB-231 cells. Goniothalamine treatment of cells significantly induced cell cycle arrest at G₂/M phase and apoptosis. By means of cell cycle synchronization, the G₂/M phase cells proved to be the most sensitive fraction to goniothalamine-induced apoptosis. Cells treated with goniothalamine revealed an increase in intracellular reactive oxygen species and a decrease in intracellular free thiol contents. The disruption of intracellular redox balance caused by goniothalamine was associated with an enhancement of cdc25C degradation. Furthermore, the antioxidant *N*-acetylcysteine and the glutathione synthesis inhibitor DL-buthionine-(*S*, *R*)-sulfoximine, inhibited and enhanced, respectively, the effects of goniothalamine on cell cycle arrest and apoptosis. Taken together, our results demonstrate for the first time that goniothalamine disrupts intracellular redox balance and induces cdc25C degradation, which in turn causes cell cycle arrest and cell death maximally at G₂/M phase in MDA-MB-231 cells.

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1. Introduction

Cell death due to apoptosis has received much attention, since many chemotherapeutic drugs have been found to induce apoptosis in a variety of tumor cell lines (Houghton, 1999; Sellers and Fisher, 1999; Mikhail, 2004). There are two major apoptotic pathways in mammalian cells (Jacobson et al., 1997; Adams and Cory, 1998). In the death receptor pathway, the activation of Fas by Fas ligand triggers initiator caspase-8/10 activation and subsequent activation of effector caspase-3. These processes result in protein cleavage, chromatin condensation, breakdown of DNA molecules, and, eventually, apoptotic cell death (Liu et al., 1997). Another apoptotic signal pathway is the mitochondrial pathway. The diverse pro-

apoptotic stimuli converging on mitochondria cause mitochondrial permeability transition, membrane potential collapse and subsequent apoptogenic proteins release. Cytochrome *c* is released into the cytosol where it triggers the formation of apoptosome, a multimeric molecule composed of apoptotic protease activating factor-1 (Apaf-1), ATP and cytochrome *c* (Cain et al., 2002). The apoptosome recruits and processes caspase-9 to form a holoenzyme complex, which in turn recruits and activates the central caspase, caspase-3. Activation of caspase-3 is often considered as the point-of-no-return in the apoptotic signaling cascade.

It has long been known that the intracellular redox status plays an important role in cell survival and death (Hampton et al., 1998; Ueda et al., 2002). The intracellular redox status is a precise balance between oxidative stress and endogenous thiol buffers present in the cell (Davis et al., 2001; Lin et al., 2003). Oxidative stress has been reported to cause a loss of intracellular glutathione (GSH) (Nordberg and Arner, 2001) which, in turn, evokes many intracellular events, such as gene activation, cell

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cycle arrest, and apoptosis (McGowan et al., 1998; Helt et al., 2001; Ueda et al., 2002). There is ample evidence that many chemotherapeutic agents such as vinblastin, etoposide, adriamycin and methotrexate elicit the apoptotic process through the generation of elevated amount of reactive oxygen species (Simizu et al., 1998; Xia et al., 1999).

The styryl-pyrone goniothalamin found within the family Annonaceae is one of the bioactive styryl-lactones which appear to be mainly restricted to the genus *Goniothalamus* (Blázquez et al., 1999). This compound can serve as an antimicrobial (Mosaddik and Haque, 2003), insecticidal (Kabir et al., 2003) and antifertility agent in rats (Azimahtol et al., 1994). In particular, it was reported to exert cytotoxic properties in a variety of tumor cell lines including MCF-7, HeLa cells (Ali et al., 1997; Hawariah and Stanslas, 1998). In HL-60 cells, it is demonstrated that goniothalamin-induced apoptosis occurs via mitochondrial pathway (Inayat-Hussain et al., 2003). However, the precise molecular mechanisms have not been clarified yet. In this study, we investigated the molecular events of goniothalamin-induced cytotoxicity in MDA-MB-231 human breast cancer cells. Our studies demonstrated that goniothalamin disrupted the intracellular redox status, and then induced cell cycle-specific apoptosis. In addition, the loss of mitochondrial membrane potential was a late and subsequent event in the apoptotic pathways.

2. Materials and methods

2.1. Drugs and chemicals

RPMI 1640 medium, fetal bovine serum, streptomycin/penicillin G were obtained from GIBCO BRL (Gaithersburg, MD). 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA), monobromobimane and rhodamine 123 were obtained from Molecular Probes (Eugene, OR). Anti-cytochrome *c* monoclonal antibody, anti-caspase-3 monoclonal antibody, anti-cdc25C monoclonal antibody and anti-poly (ADP-ribose) polymerase (PARP) monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. Z-Val-Ala-Asp (OMe)-fluoromethyl ketone (zVAD-fmk) was obtained from Calbiochem® (Biosciences, Inc). DMSO, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DL-buthionine-(*S,R*)-sulfoximine (BSO), glutathione (GSH), *N*-acetylcysteine, propidium iodide, Hoechst 33342 and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Goniothalamin (Fig. 1) was isolated from *G. amuyon* by the methods described previously (Lan et al., 2003). The purity of

goniothalamin used in our study is over 98% determined by HPLC. The DMSO stock solution was kept at –20 °C and freshly diluted to the desired concentration with cultured medium immediately before use (the final concentration of DMSO in culture medium was 0.2%).

2.2. Cell culture

MDA-MB-231 human breast cancer cells were obtained from American Tissue Culture Collection. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ in RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were harvested by trypsinization and plated 24 h before treatment with the test drugs.

2.3. Evaluation of cell viability

The cell viability was determined by the MTT assay (Mosmann, 1983). 5×10^3 cells were plated in 96-well microtiter plates and treated with the various concentrations of goniothalamin for different indicated times. At the end of each time point, 100 µl of MTT (0.5 mg/ml) were added to each well after removing the growth medium. The plates were then incubated at 37 °C for 1 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. At the end of the 1 h incubation, the MTT solution was removed, and 100 µl of DMSO were added to each well to dissolve the formazan crystals. The absorbance in individual well was determined at the absorbance at 550 nm. All of the experiments were plated in triplicate, and the results of assays were presented as means ± S.E.M.

2.4. Measurement of cell cycle/DNA content

MDA-MB-231 cells (2×10^5) were seeded onto 6 well plates and treated with the test drugs for the indicated times. Cells were harvested by trypsinization and fixed overnight in 70% ethanol at 4 °C, then collected by centrifugation and resuspended in phosphate-buffered saline (PBS) containing 25 µg/ml RNase and 0.5% Triton-X100 and incubated for 1 h at 37 °C. Finally, cells were stained with 50 µg/ml propidium iodide for 15 min at 4 °C in the dark. The relative DNA content of these cells were analyzed by a flow cytometer (Beckman Coulter, EPICS XL-MCL) based on red fluorescence. Ten thousand cells were counted and the histogram of the cell cycle distribution was analyzed by WinMDI 2.8 software. The percentage of cells with hypodiploid DNA content (subG₁) represented fraction undergoing apoptotic DNA fragmentation.

2.5. Nuclear staining with Hoechst 33342

After treatment, cells were harvested and washed with ice-cold PBS, fixed in 4% paraformaldehyde at 4 °C for 30 min. The cells were then incubated in nuclear fluorochrome Hoechst 33342 (Sigma) at a final concentration of 5 µg/ml at room

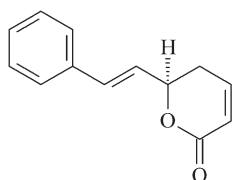


Fig. 1. Chemical structure of goniothalamin isolated from *G. amuyon*.

temperature for 30 min. Nuclear morphology was then examined with a Zeiss inverted fluorescence microscope.

2.6. Subcellular fraction for cytochrome *c* release

MDA-MB-231 cells were treated with 30 μ M goniothalamin for 0, 1, 3, 6, 12 h. Cells were harvested and washed with ice-cold PBS, and then the cell pellets were suspended in 500 μ l of buffer A (20 mM Hepes-KOH, pH 7.4, 250 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1.5 mM sodium EDTA, 1.5 mM sodium EGTA, 1 mM dithiothreitol, cocktail protease inhibitor). Cells were homogenized for 50 strokes and centrifuged at 200 $\times g$ for 10 min at 4 °C. To isolate cytosolic fraction, the supernatant was centrifuged further at 16,000 $\times g$ for 30 min at 4 °C, and the final supernatant was used as cytosolic fraction. The pellet was lysed with 100 μ l buffer B (50 mM Hepes, pH 7.4, 1% Nonidet P40, 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol, cocktail protease inhibitor) and then centrifuged at 16,000 $\times g$ for 30 min at 4 °C. The resulting supernatant was used as mitochondrial fraction. Aliquots of cytosolic or mitochondrial fractions were used for Western blot analysis of cytochrome *c*.

2.7. Assessment of mitochondrial membrane potential

The level of mitochondrial membrane potential ($\Delta\Psi_m$) was determined by flow cytometry after staining with rhodamine 123, a cationic fluorochrome with lipophilic property whose distribution to the mitochondria matrix correlates with the $\Delta\Psi_m$ (Sureda et al. 1997). After treatment, cells were loaded with 10 μ M rhodamine 123 and incubated at 37 °C for 30 min in the dark. Cells were then harvested, washed and resuspended in PBS for flow cytometry assay with the excitation wavelength at 488 nm and the emission wavelength at 525 nm.

2.8. Western blot assay

To prepare whole cell lysates, MDA-MB-231 cells were harvested after scraping and then centrifuged at 200 $\times g$ for 5 min. Cell pellets were homogenized by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 20 μ g/ml leupeptin, 2 mM sodium orthovanadate, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 5 mM sodium fluoride, and 5000 U/ml aprotinin). After the cell suspensions were centrifuged at 16,000 $\times g$ for 10 min at 4 °C, the supernatants were collected and stored at –20 °C. The protein concentration was determined with the Bradford method (Bio-Rad) as described in manufacturer's manual. Equal amounts of proteins (100 μ g/lane) were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. After blocking with TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% (w/v) non fat milk at 4 °C overnight, the membranes were incubated for 1 h at room temperature with the primary antibodies against cdc25C, caspase-3, or PARP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with Tris-buffered saline with Tween 20 (TBS-T), the membranes were incubated with goat anti-mouse IgG-

horseradish peroxidase secondary antibody (1:2000) for 1 h at room temperature. Finally, the signals were detected by enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

2.9. Detection of intracellular reactive oxygen species accumulation

Intracellular reactive oxygen species accumulation was monitored using H_2DCFDA , which is a relative specific probe for the presence of hydrogen peroxide (LeBel and Bondy, 1990). At the end of the treatments, cells were loaded with 20 μ M H_2DCFDA and incubated at 37 °C for 30 min in the dark. Cells were then collected, washed and resuspended in PBS and analyzed immediately using flow cytometry with the excitation and emission wavelengths of 490 and 530 nm, respectively.

2.10. Determination of intracellular free thiol levels

The content of intracellular free thiols was determined using monobromobimane, which passively diffuses across the plasma membrane into the cytoplasm where it forms blue fluorescent adducts with the reduced form of GSH and other thiol-containing proteins (Aoshiba et al., 1999). Briefly, after treatments, cells in 96-well plates were loaded with 100 μ M monobromobimane for 30 min in the dark. Then, plates were read on a micro-plate reading fluorometer (Fluostar®, BMG Co.) using excitation and emission wavelengths of 390 and 460 nm, respectively.

2.11. Statistics

Data are presented as means $\pm$ S.E.M. and comparisons were made using Student's *t* test. A probability of 0.05 or less was considered statistically significant.

3. Results

3.1. Inhibition of cell viability by goniothalamin in MDA-MB-231 cells

We examined the effect of goniothalamin on the cell viability of MDA-MB-231 human breast cancer cells using a conventional tetrazolium-based (MTT) assay. The results shown in Fig. 2 reveal a dose- and time-dependent inhibition of cell viability. After 72 h, goniothalamin inhibited completely the cell viability with an IC_{50} value of about 1.46 μ M. The decreased cell viability could be the result of the inhibition of growth and/or the induction of apoptosis. We therefore investigated whether goniothalamin could induce cell cycle arrest and apoptosis in breast cancer cells.

3.2. Effects of goniothalamin on cell cycle distribution

The ploidy state of MDA-MB-231 cells was monitored by flow cytometry after propidium iodide staining nuclei. DNA histogram showed that goniothalamin increased the population

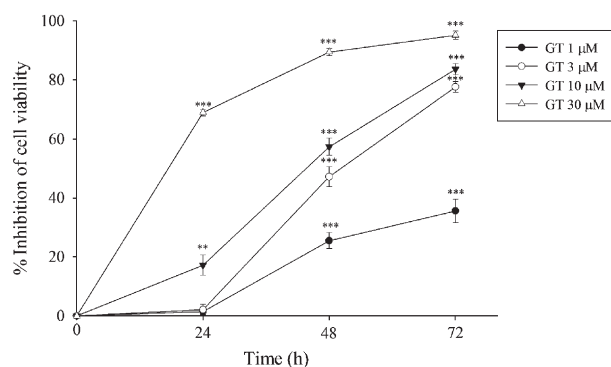


Fig. 2. Effect of goniiothalamine on cell viability in MDA-MB-231 human breast cancer cells. Cells were treated with DMSO or variant concentrations of goniiothalamine for 24, 48 or 72 h. Cell viability was measured by MTT assay as the absorbance at 550 nm. Results are presented as means $\pm$ S.E.M. of 3–4 independent experiments. ** $P < 0.01$, *** $P < 0.001$ as compared with the control.

of cells at G₂/M phase in a time-dependent manner (Fig. 3). The G₂/M population increased significantly from $22.96 \pm 0.98\%$ in the control to $33.68 \pm 1.25\%$ in cells treated with 30 μ M goniiothalamine for 12 h, while concomitantly the G₁ population decreased from $50.69 \pm 1.94\%$ to $40.71 \pm 2.97\%$. The percentage of S phase cells was not profoundly affected. These results indicated that goniiothalamine could induce G₂/M phase arrest in MDA-MB-231 cells. Concurrently, goniiothalamine induced a concentration- and time-dependent increase in the proportion of subG₁ population (Fig. 4A), suggesting that the cells underwent DNA fragmentation which is a biochemical hallmark of apoptosis. The apoptotic cells were further evidenced by nuclear staining with fluorochrome Hoechst 33342. In the absence of goniiothalamine, cells presented nuclei with homogenous chromatin distribution (Fig. 4B, left panel). However, goniiothalamine induced chromatin condensation and nuclear fragmentation (Fig. 4B right panel, arrows).

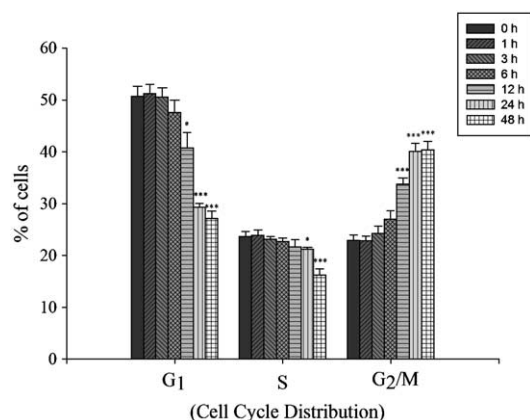


Fig. 3. Effect of goniiothalamine on cell cycle distribution. MDA-MB-231 cells were treated with 30 μ M goniiothalamine for the indicated times. The cell cycle distribution was examined with propidium iodide fluorescence by flow cytometry as described in “Materials and methods”. Results are presented as means $\pm$ S.E.M. of 4 independent experiments. * $P < 0.05$, *** $P < 0.001$ as compared with the respective control.

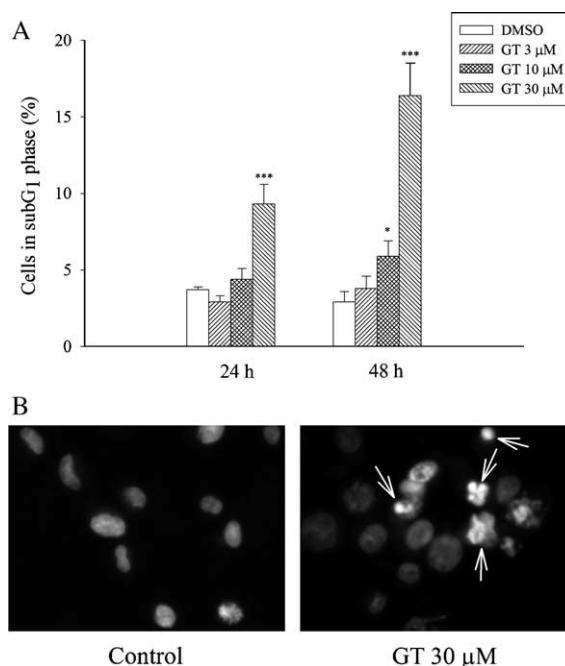


Fig. 4. Goniiothalamine induces apoptosis in MDA-MB-231 cells. (A) To determine the apoptotic cells, MDA-MB-231 cells were treated with DMSO or 3, 10, 30 μ M goniiothalamine for 24 or 48 h. Apoptosis was determined by flow cytometry analysis of DNA fragmentation of propidium iodide-stained nuclei. Results are presented as mean $\pm$ S.E.M. of 3–5 independent experiments. * $P < 0.05$, *** $P < 0.001$ as compared with the respective control. (B) To observe the chromatin condensation, cells were treated with DMSO or 30 μ M goniiothalamine for 48 h. Then, the cells were harvested and stained with Hoechst 33342 (5 μ g/ml) as described in “Materials and methods”. Nuclear morphology was examined with a Zeiss fluorescence microscope. Magnification is $\times 400$. Arrows indicate condensed or fragmented nuclei. Results are representative of 3 independent experiments.

3.3. Goniiothalamine induces cytochrome *c* release and caspase-3 activation

To investigate further the mechanism of goniiothalamine-induced apoptosis, the release of cytochrome *c* from mitochondria was measured. As shown in Fig. 5, a time-dependent accumulation of cytochrome *c* in the cytosol was detected in goniiothalamine-treated cells, and the efflux in mitochondria was observed simultaneously. It has been suggested that translocation of cytochrome *c* from the mitochondria is an important event in apoptotic signaling, which subsequently causes apoptosis by activation of caspase-9 in the presence of Apaf-1, thus resulting in the activation of downstream caspase-3 (Susin et al., 1999). Therefore, we determined the levels of caspase-3 and the cleavage of its substrate, PARP, which is a DNA repair enzyme. Activation of caspase-3 from the p32 proform to its active subunits, p24/p17, and the cleavage of PARP from the p116 proform to its subunits, p85 was concurrently observed after 6 h of goniiothalamine treatment (Fig. 6A). Pretreatment of cells with 50 μ M zVAD-fmk, a general and potent inhibitor of caspases, attenuated significantly goniiothalamine-induced apoptosis (Fig. 6B). However, the G₂/M phase arrest caused by goniiothalamine ($48.2 \pm 1.0\%$) was not prevented by zVAD-fmk ($51.3 \pm 3.5\%$). These results suggested that

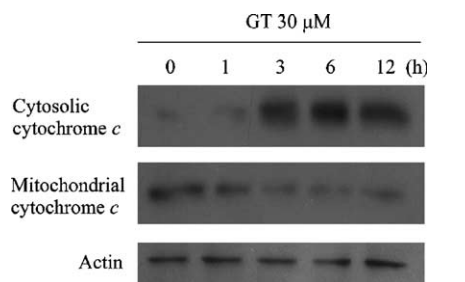


Fig. 5. Goniiothalamine causes cytochrome *c* release from mitochondria. MDA-MB-231 cells were treated with 30 μM goniiothalamine for the indicated times. Cytosolic and mitochondrial fractions were isolated as described in “Materials and methods”. Cytochrome *c* was determined by Western blot assay using cytochrome *c* antibody.

goniiothalamine induced an efflux of cytochrome *c* from mitochondria and caused caspase-3 dependent apoptosis in MDA-MB-231 cells in addition to perturbation of cell cycle progression.

3.4. Goniiothalamine induces cell cycle-specific apoptosis

To analyze whether goniiothalamine-induced apoptosis is cell cycle-specific, we synchronized MDA-MB-231 cells at G₁, S, or G₂ phase by pretreatment with the specific cell cycle

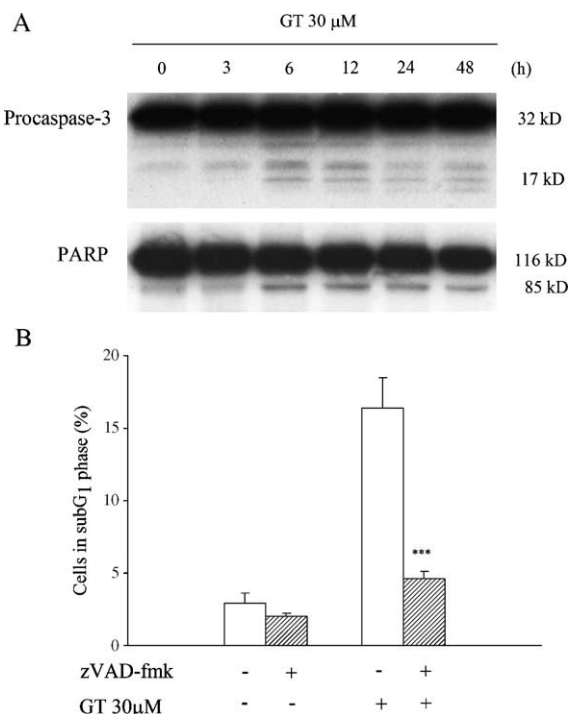


Fig. 6. Goniiothalamine induces caspase-mediated apoptosis. (A) Effect of goniiothalamine on the cleavage of pro-caspase-3 and PARP. Cells were treated with 30 M goniiothalamine and harvested at the indicated times. Equal amount of protein (100 μg/lane) were subjected to SDS–PAGE and analyzed by Western blot. (B) Effect of the caspase inhibitor, zVAD-fmk, on goniiothalamine-induced apoptosis. MDA-MB-231 cells were pretreated with 50 μM zVAD-fmk for 1 h before challenged with 30 μM goniiothalamine for 48 h. Apoptosis was determined with propidium iodide fluorescence by flow cytometry. Results are presented as mean±S.E.M. of 4–5 independent experiments. ****P*<0.001 as compared with the respective control.

Table 1
Effects of various cell cycle inhibitors on the cell cycle distribution

	G ₁ phase	S phase	G ₂ /M phase
DMSO	45.58±0.77	27.10±1.09	27.33±1.15
Mimosine	51.08±1.41*	29.58±1.27	19.33±0.69***
Thymidine	38.80±1.36***	41.25±1.60***	19.95±0.64***
Nocodazole	8.18±1.52***	6.03±1.51***	85.50±1.15***

MDA-MB-231 cells were pretreated with 0.4 mM mimosine, 2 mM thymidine for 24 h, or 0.2 μg/ml nocodazole for 12 h, respectively. The cell cycle distribution was examined with propidium iodide fluorescence by flow cytometry as described in “Materials and methods”. Results are presented as means±S.E.M. of 4 independent experiments. **P*<0.05, ****P*<0.001 as compared with the respective control.

inhibitors, mimosine (0.4 mM) and thymidine (2 mM) for 24 h, or nocodazole (0.2 μg/ml) for 12 h, respectively (Table 1). Then, these cells were further treated with goniiothalamine for another 24 h. As shown in Fig. 7, arresting cells in G₂/M phase by nocodazole sensitized cells for subsequent treatment with goniiothalamine. The apoptotic cells induced by nocodazole alone were 6.80±0.63% whereas that induced by the combination with goniiothalamine were 19.15±1.68%. In contrast, pretreatment of mimosine or thymidine had no or little effects on goniiothalamine-induced apoptosis. Moreover, no marked increases in the proportion of subG₁ cells population were observed when cells were synchronized at G₀/G₁ phase by starving with serum-free medium for 24 h before goniiothalamine treatment (data not shown). These findings indicated a differential sensitivity for goniiothalamine-induced apoptosis at different phases of the cell cycle.

3.5. Goniiothalamine induces change of redox status in MDA-MB-231 cells

Oxidative stress induces a variety of cellular responses including apoptosis (Nakamura et al., 1997; Ueda et al., 1998; Fernandez-Checa, 2003). It is thus interesting to know

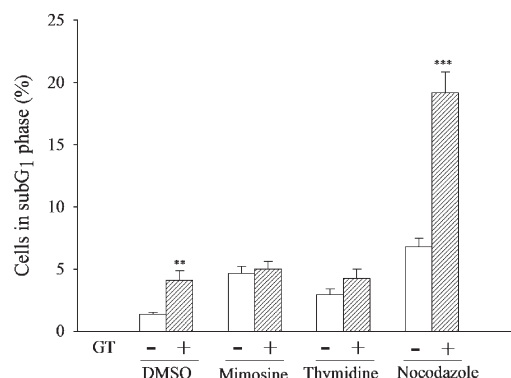


Fig. 7. Effects of cell cycle inhibitors on goniiothalamine-induced apoptosis. MDA-MB-231 cells were pretreated with 0.4 mM mimosine, 2 mM thymidine for 24 h, or 0.2 μg/ml nocodazole for 12 h, respectively, and further cultured in the presence or absence of 30 μM goniiothalamine for another 24 h. Apoptosis induced by the combination with goniiothalamine and the respective cell cycle inhibitor was determined with propidium iodide fluorescence by flow cytometry. Results are presented as mean±S.E.M. of 4 independent experiments. ***P*<0.01, ****P*<0.001 as compared with the respective control.

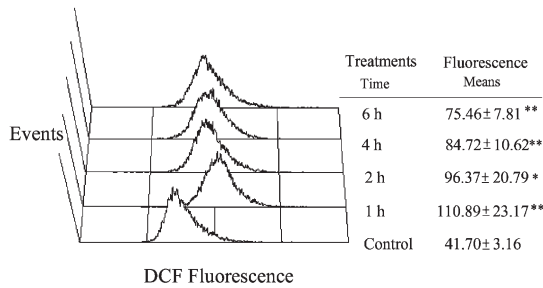


Fig. 8. Effect of goniothalamin on intracellular reactive oxygen species formation in MDA-MB-231 cells. Cells were treated with DMSO or 30 μ M goniothalamin for up to 6 h. After treatments, cells were loaded with H_2DCFDA (20 μ M) and further incubated for another 30 min prior to flow cytometry analysis. Results are representative of 3 independent experiments. * P <0.05, ** P <0.01 as compared with the DMSO control.

whether goniothalamin may induce oxidative stress on MDA-MB-231 cells. We use the H_2DCFDA -derived fluorochrome as an indicator of peroxides and superoxide accumulation. A relative low level of reactive oxygen species was observed in control cells whereas marked formation of intracellular reactive oxygen species was observed after the addition of goniothalamin (30 μ M) for up to 6 h. (Fig. 8). In particular,

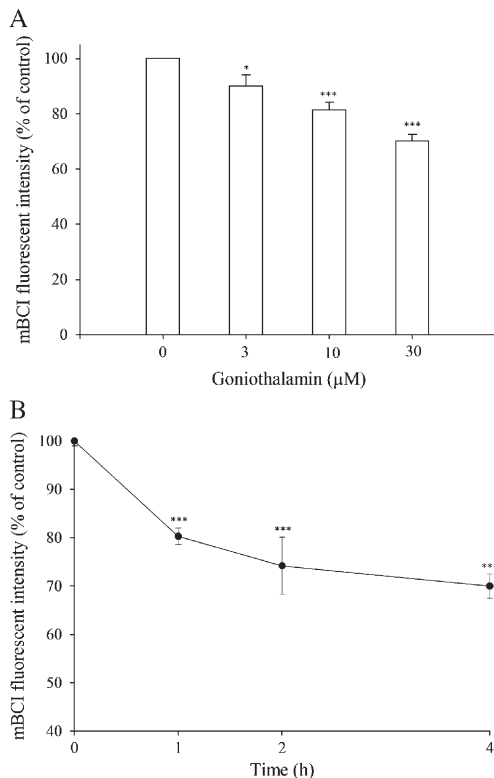


Fig. 9. Effect of goniothalamin on intracellular thiol levels. (A) Cells were treated with DMSO or 3, 10, 30 μ M goniothalamin for 4 h. Then, the cells were loaded with monobromobimane (200 μ M) and further incubated for another 30 min. Fluorescence intensity was detected by a micro-plate reading fluorometer. (B) Cells were treated with 30 μ M goniothalamin for the indicated times. After treatment, cells were loaded with monobromobimane and the fluorescence intensity was measured as the above described. Results are presented as mean $\pm$ S.E.M. of 4–6 independent experiments. * P <0.05, *** P <0.001 as compared with the respective control.

the 2',7'-dichlorofluorescein (DCF) fluorescence intensity detected in the goniothalamin-treated cells was about 3 times higher than that in the control cells at the first hour of the treatment period. To confirm whether the redox state in the goniothalamin-treated cells was changed, we analyzed the total contents of intracellular GSH and protein thiol. Cells were incubated with monobromobimane, as an indicator of cellular free thiol, and then analyzed by micro-plate reading fluorometer. Goniothalamin-induced thiol depletion was found to be dose-(Fig. 9A) and time-dependent (Fig. 9B). In particular, a significant decrease in thiol contents was observed as early as 1 h after the addition of goniothalamin. These findings demonstrated that goniothalamin increased the intracellular reactive oxygen species formation concomitantly with the decrease in intracellular thiol contents in MDA-MB-231 cells.

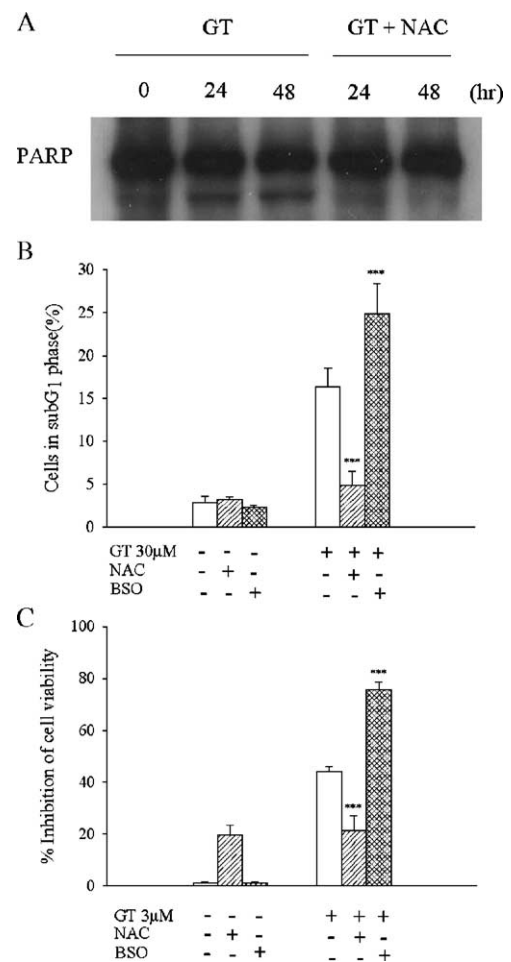


Fig. 10. Effects of *N*-acetylcysteine or BSO on goniothalamin-induced responses in MDA-MB-231 cells. (A) Cells were incubated with 10 mM *N*-acetylcysteine for 3 h before treated with 30 μ M goniothalamin for the indicated times. The cleavage of PARP was determined by Western blot. (B) Cells were incubated with 10 mM *N*-acetylcysteine or 250 μ M BSO for 3 h before treated with 30 μ M goniothalamin for 48 h. The proportion of cells in subG1 phase was determined with propidium iodide fluorescence by flow cytometry. (C) Cells were treated as mentioned above. The percentage inhibition of cell viability was determined by MTT assay. Results are presented as mean $\pm$ S.E.M. of 3–5 independent experiments. *** P <0.001 as compared with the respective control.

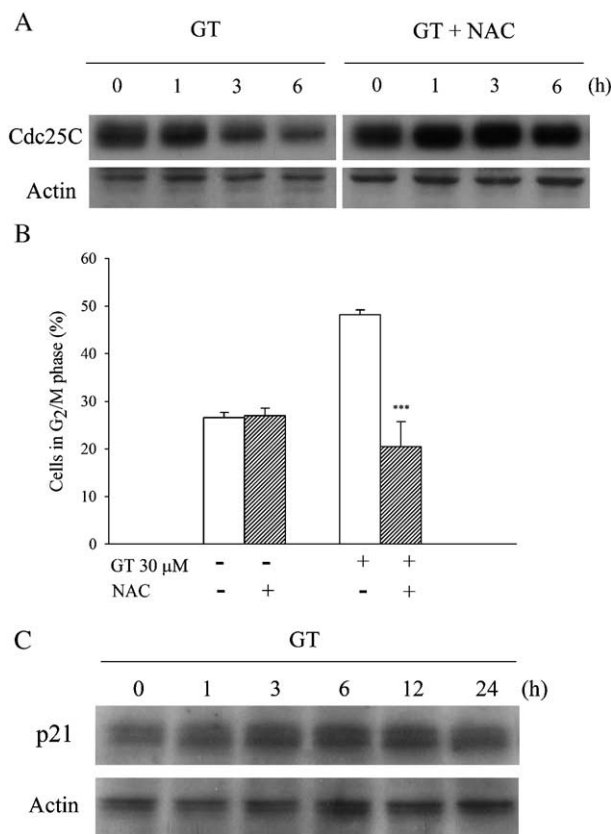


Fig. 11. Expression of the cell cycle regulators and the effect of *N*-acetylcysteine on the G₂/M phase arrest induced by goniothalamin. (A) MDA-MB-231 cells were untreated or pretreated with 10 mM *N*-acetylcysteine for 3 h before treatment with 30 μM goniothalamin for the indicated times. The levels of cell cycle regulators, cdc25C, were determined by Western blot. (B) Cells were treated as mentioned above for 48 h. The proportion of cells in G₂/M phase was determined with propidium iodide fluorescence by flow cytometry. Results are presented as mean±S.E.M. of 3–5 independent experiments. ****P*<0.001 as compared with the respective control. (C) Cells were treated with 30 μM goniothalamin for the indicated times. The levels of cell cycle regulators, p21, were determined by Western blot.

3.6. Effect of antioxidants and BSO on goniothalamin-induced changes of MDA-MB-231 cells

We next examined the effects of antioxidants on goniothalamin-induced responses in MDA-MB-231 cells. Pretreatment of cells with *N*-acetylcysteine prevented goniothalamin-induced PARP cleavage, apoptosis, and the decrease in cell viability (Fig. 10). We had similar results with cells treated with GSH before goniothalamin was added (data not shown). These results revealed that goniothalamin-induced apoptosis and cytotoxicity were dependent on pro-oxidant-mediated mechanism.

Pretreatment of DL-buthionine-(*S,R*)-sulfoximine (BSO), a specific inhibitor of γ-glutamyl-cysteine synthetase, offered an approach to studying the effect of GSH deficiency (Martensson et al., 1991). Intracellular thiol contents were decreased by 30.3±2.3% and 38.8±3.4% in cells after 48 h of 250 μM BSO and 30 μM goniothalamin treatment, respectively. However, treatment of goniothalamin after BSO pretreatment further depleted the intracellular thiol contents (66.8±1.7%).

We then investigated the influences of BSO on apoptosis and cell viability in goniothalamin-treated cells. Pretreatment of the nontoxic concentration of BSO strongly cooperated with goniothalamin to induce apoptosis and enhance the inhibition of cell viability (Fig. 10B and C). Taken together, these results indicated that the depletion of intracellular thiols plays a role in the cell death caused by goniothalamin.

3.7. Effect of goniothalamin on cell growth regulatory proteins

Reactive oxygen species are known to interfere with the cell cycle (Helt et al., 2001). It was also reported that oxidative stress may induce cell cycle arrest in part through the degradation of cdc25C (Savitsky and Finkel, 2002). Accordingly, we determined the expression of cdc25C in the goniothalamin-treated cells by Western blot. When MDA-MB-231 cells were treated with goniothalamin, a significant decrease in cdc25C levels was detectable as early as 3 h after treatment (Fig. 11A, left panel). Goniothalamin-induced cdc25C degradation and G₂/M phase arrest were also prevented by *N*-acetylcysteine (Fig. 11A, right panel, and B). These results revealed that goniothalamin induced the change in intracellular redox status which, in turn, caused the degradation of cdc25C and thus led to G₂/M phase arrest. In contrast to cdc25C, the

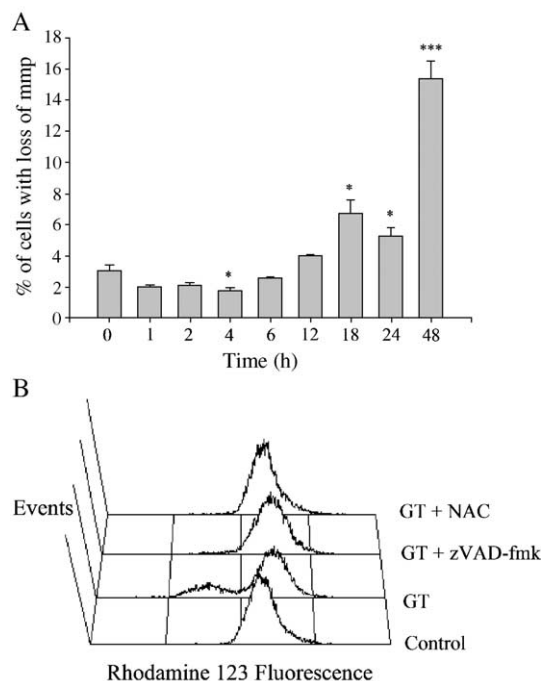


Fig. 12. Effect of *N*-acetylcysteine or zVAD-fmk on goniothalamin-induced loss of mitochondrial membrane potential (mmp) in MDA-MB-231 cells. (A) Cells were treated with 30 μM goniothalamin for the indicated times. At the end of the incubation time, cells were then loaded with 10 μM rhodamine 123 for 30 min, followed by flow cytometry analysis. Results are presented as mean±S.E.M. of 3 independent experiments. **P*<0.05, ****P*<0.001 as compared with the DMSO control. (B) Cells were pretreated with zVAD-fmk (50 μM) for 1 h or *N*-acetylcysteine (10 mM) for 3 h, respectively, and further treated with 30 μM goniothalamin for another 48 h. The fluorescence intensity of rhodamine 123 was determined as described above. Results are representative of 3 independent experiments.

expression of another important inhibitor of cell cycle, namely p21, was not significantly affected in goniiothalamine-treated cells (Fig. 11C).

3.8. Goniiothalamine induced loss of mitochondria membrane potential

A decrease in mitochondrial membrane potential ($\Delta\Psi_m$) is associated with dysfunction of mitochondria and subsequent apoptotic cell death (Zamzami et al., 1995; Barbu et al., 2002). Since oxidative stress and GSH depletion may impair mitochondrial function leading to cytochrome *c* release that is central to cellular apoptosis (Hancock et al., 2001), we therefore examined whether goniiothalamine has an impact on $\Delta\Psi_m$. Cells were treated with goniiothalamine for various incubation periods and then stained with rhodamine 123 before being harvested. With its high negative charges, rhodamine 123 will accumulate in normal mitochondria whereas the reduction of $\Delta\Psi_m$ will lead to the release of the dye and the reduction of its fluorescent intensity. As seen in Fig. 12, the collapse in $\Delta\Psi_m$ was detectable significantly after 18 h of incubation with goniiothalamine (Fig. 11A), and that was completely inhibited by pretreatment of zVAD-fmk or *N*-acetylcysteine (Fig. 12B).

4. Discussion

In this study we showed that the decrease in cell viability by goniiothalamine was due to induction of cell cycle arrest at the G₂/M phase and apoptosis in the human breast cancer cell line, MDA-MB-231. The apoptosis induced by goniiothalamine was evidenced by cytochrome *c* release from mitochondria, caspase-3 activation, PARP cleavage, chromatin condensation, and DNA fragmentation. Furthermore, the caspase inhibitor, zVAD-fmk, inhibited goniiothalamine induced apoptosis. These results indicated that goniiothalamine-induced apoptosis involved a caspase-mediated mechanism. It is well known that cytochrome *c* released during apoptosis binds to the adaptor molecule Apaf-1, and leads to the activation of caspase-9 through the formation of the multimeric apoptosome complex. Caspase-9 then activates the central caspase in the apoptotic signaling machinery, caspase-3 (Cain et al., 2002). Further studies need to clarify whether other upstream caspases might be involved (e.g., caspase-2). Taken together, goniiothalamine-induced apoptosis in MDA-MB-231 cells, at least partly, was through the mitochondrial pathway.

To examine the relationship between goniiothalamine-induced G₂/M phase arrest and apoptosis, we found the following. (1) When cells synchronized at G₀ phase by serum deprivation were treated with goniiothalamine and completed medium, no marked increase in apoptotic cells were observed. (2) Treatment of nocodazole, but not mimosine or thymidine, predisposed cells to apoptotic signals induced by goniiothalamine. (3) Pretreatment of zVAD-fmk blocked apoptosis but not G₂/M phase arrest induced by goniiothalamine. These results suggest that goniiothalamine-induced apoptosis is cell cycle-specific and, cells at G₂/M phase are most susceptible to goniiothalamine.

Cancer cells, particularly those that are highly invasive or metastatic, may require a certain level of oxidative stress to maintain a balance between undergoing either proliferation or apoptosis (Toyokuni et al., 1995; Loo, 2003). While the constitutive high production of H₂O₂ in some cancer cells appears to promote their proliferation, additional amounts of H₂O₂ above a certain threshold cause cell cycle arrest and/or apoptosis (Loo, 2003; Kurata, 2000; Kim et al., 2001). To explore the underlying mechanism associated with the goniiothalamine-induced responses in MDA-MB-231 cells, a highly invasive breast cancer cell line, we studied the possible involvement of goniiothalamine-mediated oxidative stress in the apoptotic process. We found that goniiothalamine could indeed generate elevated levels of reactive oxygen species in the early stage of the apoptotic process. Besides the generation of the intracellular reactive oxygen species, we also found that intracellular thiol declined rapidly in a dose- and time-dependent manner. To explore the meaning of reactive oxygen species formation and GSH depletion in goniiothalamine-treated MDA-MB-231 cells, we used the general antioxidant *N*-acetylcysteine, which serves both as a redox buffer and a reactive oxygen intermediate scavenger (Deneke, 2000), and the inhibitor of GSH synthesis, BSO. *N*-acetylcysteine prevented goniiothalamine-induced cell cycle arrest and cell death whereas BSO enhanced the responses to goniiothalamine. These results clearly indicated that reactive oxygen species production and/or GSH depletion play an important role in goniiothalamine-induced cell cycle arrest and apoptosis.

The G₂/M transition is characterized by phosphoregulation of Cdc2 kinase, a key enzyme, which when bound to its partner cyclin B, drives cells into mitosis. Entry into mitosis requires dephosphorylation of Cdc2 by cdc25 phosphatases (Taylor and Stark, 2001; Bunz et al., 1998; Kastan, 2001). It has been reported that oxidative stress may induce cell cycle arrest through degradation of cdc25C (Savitsky and Finkel, 2002; Zhang et al., 2003). In agreement with previous research, the results obtained from the present study showed that treatment of goniiothalamine led to degradation of cdc25C following the increase in intracellular reactive oxygen species and GSH depletion. Furthermore, both cdc25C degradation and G₂/M phase arrest caused by goniiothalamine were prevented by *N*-acetylcysteine. These data demonstrated that goniiothalamine induced oxidative stress and which, in turn, caused cdc25C degradation preceding G₂/M phase arrest in MDA-MB-231 cells. Additionally, the G₂/M arrest after goniiothalamine treatment was p53-independent since these cells possess a mutation in the p53 gene (codon 280, AGA to AAA, Arg to Lys) (Li et al., 1999). p21, a downstream protein of p53, is a universal Cdk inhibitor and caused cell cycle arrest at G₁/S or G₂/M (Sherr and Roberts, 1995; Tchou et al., 1996). In addition to direct transcriptional induction by p53, recent studies have demonstrated that p21 was also induced independently from p53 by a variety of signals (Michieli et al., 1994; Li et al., 1999). However, there was no significant change in the level of p21 in goniiothalamine-treated cells. Taken together, these results suggested that goniiothalamine regulated cdc25C levels, but not p21, and thus induced G₂/M phase arrest.

Mitochondria are particularly affected in the early apoptotic process and are now thought to act as central coordinators of cell death (Green and Reed, 1998; Henry-Mowatt et al., 2004). Mitochondrial dysfunction could induce opening of the mitochondrial permeability transition pore (PTP), dissipation of $\Delta\Psi_m$, and release of apoptogenic proteins (cytochrome *c* and AIF) (Buttke and Sandstrom, 1994). In many cases of apoptotic induction, a loss of $\Delta\Psi_m$ is required before a complete loss of cytochrome *c* from the mitochondria (Zamzami et al., 1995; Barbu et al., 2002; De Giorgi et al., 2002). However, there is also accumulating evidence that $\Delta\Psi_m$ loss can be a late and subsequent event in the apoptotic pathway. For example, the loss of $\Delta\Psi_m$ could only be detected after the cytochrome *c* release, caspase activation and DNA fragmentation in staurosporine-treated HL-60 cells, but not Jurkat cells (Finucane et al., 1999). The possible mechanism has been described that pro-apoptotic Bcl-2 superfamily members, Bax or Bid, were shown to form conducting channels in the outer mitochondrial membrane. These channels are large enough for the release of cytochrome *c* into the cytosol (Minn et al., 1997; Schendel et al., 1997). On the other hand, compounds which directly dissipate the $\Delta\Psi_m$, such as CCCP, do not rapidly induce apoptosis (Finucane et al., 1999). These data indicated that the release of cytochrome *c*, and not the loss of $\Delta\Psi_m$, is a step that initiates the cell death program, imply that the latter may be cell type or inducer specific feature of apoptosis. In agreement, we found that cytochrome *c* release and caspase activation occurred before the loss of $\Delta\Psi_m$, and moreover, zVAD-fmk completely prevented goniiothalamine-induced decrease in $\Delta\Psi_m$. This indicates that the loss of $\Delta\Psi_m$ occurs downstream of caspase activation. Our results are consistent with those of Inayat-Hussain et al. (2003) in that the loss of $\Delta\Psi_m$ in goniiothalamine-treated HL-60 cells was also inhibited by zVAD-fmk. It has been recently shown that caspase-3 activated after the release of cytochrome *c* from mitochondria disrupted electron transport complexes I and II, resulting in loss of $\Delta\Psi_m$ (Fernandez-Checa, 2003; Ly et al., 2003). Therefore, these data suggested that goniiothalamine-induced caspase activation and cytochrome *c* release in MDA-MB-231 cells are not dependent on the reduction of $\Delta\Psi_m$, which may be a consequence of the apoptotic signaling pathway.

In conclusion, our result demonstrates for the first time that goniiothalamine, a natural compound with low molecular weight, disrupts intracellular redox balance and enhances cdc25C degradation and in turn causes cell cycle arrest and cell death maximally at G₂/M phase in MDA-MB-231 cells. Moreover, the collapse of mitochondrial membrane potential is not a prerequisite event for goniiothalamine-induced apoptosis. These results may give some new insight into the understanding of chemotherapeutic properties of goniiothalamine.

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