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GONIOTHALAMIN-INDUCED APOPTOSIS IN HUMAN OVARIAN CANCER CELL LINE, CAOV-3 THROUGH THE REGULATION OF BCL-2 AND BAX

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ABSTRACT. *The purpose of this study was to investigate the ability of goniothalamin to induce apoptosis by regulating Bax and Bcl-2 expression in human ovarian cell line, Caov-3. Goniothalamin is a styrylpyrone derivative that extracted from the plant Goniothalamus sp. Anti-proliferation assay showed that goniothalamin was able to inhibit proliferation of Caov-3 (EC_{50}= 1.8 μM) without causing any effect on normal kidney cell line, MBDK. To examine the ability of goniothalamin to induce apoptosis, TUNEL assay was carried out. Apoptotic Index for treated cells were 73% (10^{-6}M), 56% (10^{-7}M), and 42.2% (10^{-8}M) compared to untreated cells (23%). To ascertain the mechanisms of apoptosis regulation by Bax and Bcl-2, protein expression was studied. From immunocytochemistry and Western blot analysis, we noticed that Bax expression had increased after treatment; on the other hand suppression of anti-apoptotic protein, Bcl-2 had occurred significantly in Caov-3.*

KEYWORDS. Apoptosis, Bcl-2, Bax, Goniothalamin

INTRODUCTION

Apoptosis plays an important role in normal development, cell differentiation and tissue homeostasis (Zhang *et al.*, 1999). Dysregulation of apoptosis will result of the pathogenesis of cancer, for example resistance towards apoptosis in malignant lymphoma has caused the tumor development (Vaux *et al.*, 1988). Apoptosis can be distinguished from necrosis by morphological criteria, including membrane blebbing, chromatin condensation, and presence of apoptotic bodies and fragmentation of the genomic DNA (Zhang *et al.*, 1999).

The cells response towards the signal of apoptosis depends on the various regulatory checkpoints in the cell. One of these important regulators is Bcl-2 family protein, whose members include suppressors (Bcl-2, Bcl-X_L, Mcl-1) and proapoptotic inducers (Bax, Bak, Bcl-X_s) of cell death (Goping *et al.*, 1998). The relationships among these family members are complex. Immunoprecipitation and yeast two-hybrid select studies showed that Bax may form homodimers or heterodimers with either Bcl-2 or Bcl-X_L (Hsu & Youle, 1997). By Bax homodimers formation apoptosis can be triggered, whereas the heterodimers of Bax with Bcl-2 will counter the direction

of this phenomenon. Therefore, it has been suggested that the ratio of Bax homodimers to heterodimers may be a crucial step to initiate the apoptosis (Yang *et al.*, 1995).

Regulation of apoptosis may cause resistance to chemotherapy and affect the cancer treatment. It is commonly happened due to the development of drug resistance in tumor cells. One of the mechanisms of drug resistance is the suppression of apoptosis after cytotoxic induction (Jones *et al.*, 1998). Previous studies showed that the expression and activation of p53 and Bcl-2 protein family are important in regulating apoptosis pathway by modulating the chemosensitivity of tumor cells. Consequently, the effort of finding apoptosis-inducing drug has been widely carried out.

In this study, the research on goniothalamin's capability to promote apoptosis has been done. Goniothalamin is a styrylpyrone derivative (SPD) extracted from the plant *Goniothalamus sp*. *In vitro* studies showed that goniothalamin has selective antiproliferative action on a broad range of cancer cell lines (Azimahtol *et al.*, 1998). This has fostered an interest to investigate the ability of goniothalamin to induce apoptosis by regulating Bax and Bcl-2 expression.

MATERIALS AND METHODS

Proliferation Assay

Caov-3 and MDBK cells were cultured overnight onto a 96-well plate, and then treated with goniothalamin, tamoxifen and taxol. After 3 days incubation at 37°C, the survival cells were estimated using the methylene blue method modified from Lin and Hwang (1991). The EC_{50} value was calculated based on the drug concentration in reducing 50% of optical density.

Apoptotic Index

Caov-3 were grown on cover slips and treated with various concentrations of goniothalamin (10^{-6}M, 10^{-7}M and 10^{-8}M). After 24-hour of incubation, the presence of DNA fragmentation that indicated the occurrence of apoptosis was determined by using TUNEL assay (Promega USA). Nuclear morphology was examined with fluorescence microscope (Zeiss). The percentage of apoptotic cells was calculated from fifteen random microscopic fields at 1000x magnification.

Immunocytochemistry Assay of Bax

The cells were fixed on the slides were permeabilized with 0.2% Triton-X 100 for 20 minutes and blocked with 5% fetal calf bovine in phosphate-buffer saline (PBS) for 2 hours at 37°C. After washing with PBS for a few times, the cells were incubated overnight with anti-Bax antibody (diluted 1:250) at 4°C. Then secondary antibody conjugated with FITC was added and incubated at 37°C for 1 hour. After washing, the slides were visualized by using fluorescence microscope.

Western Blotting of Bel-2

Protein was extracted from treated and control cells, then were separated on 12% SDS-PAGE gel. After electrophoresis, protein was transferred onto nitrocellulose membrane. The membrane was dried, preblocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated with primary antibody of Bcl-2 (Pharmingen) diluted 1:1000 and mouse anti-IgG - conjugated with horseradish peroxidase diluted 1: 10,000. The blot was exposed on x-ray film.

RESULTS

Effect of Goniothalamin on proliferation of Caov-3 cells

From Table 1, we found that goniothalamin showed a selective anti-proliferative effect on ovarian cancer cells, without inhibiting the growth of normal kidney cells. In this experiment, tamoxifen and taxol had been used as positive controls since they are common anti-cancer drugs. EC_{50} value represented both the growth inhibitory (cytostatic) and cytotoxic effect on cell viability. Therefore, lower value of $EC_{50,}$ the higher potential of certain bioactive compound as an anti-cancer drug. Goniothalamin was more effective than tamoxifen by having 3-fold lower EC_{50} value. Although taxol was markedly decreased the growth of Caov-3, it also caused the death of normal cells.

Table 1. EC_{50} values of ovarian cancer cell line and normal kidney cell line after goniothalamin, tamoxifen and taxol treatments. EC_{50} was obtained from 4 individual experiments (4n), where n=3.

Cell Lines	Goniothalamin (μM)	Tamoxifen (μM)	Taxol (μM)
Caov-3	1.8	5.66	0.087
MDBK	-	6.84	0.1

Goniothalamin induced apoptosis in Caov-3 cells

The results obtained from TUNEL assay showed that goniothalamin was capable to induce the occurrence of apoptosis in Caov-3 cells. By visualizing the cells treated with goniothalamin under fluorescence microscope, fragmentation DNA generated 3-OH end, which can be labeled with fluorescein-12-dUTP (Figure 1). Then Apoptotic Index was calculated by counting the number of cells showed the fluorescence signals from the total number of cells.

Figure 2 showed that Apoptotic Index for treated Caov-3 cells after 24-hour were 73%, 56%, and 42.2% when the goniothalamin concentrations were 10^{-6}M, 10^{-7}M and 10^{-8}M respectively.

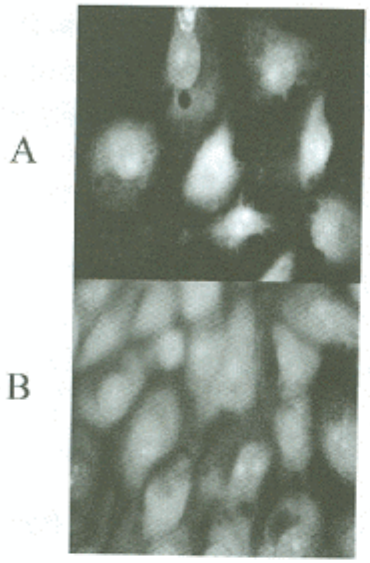

Figure1. TUNEL labeling of Caov-3. Goniothalamin treated (A) and untreated cells (B).

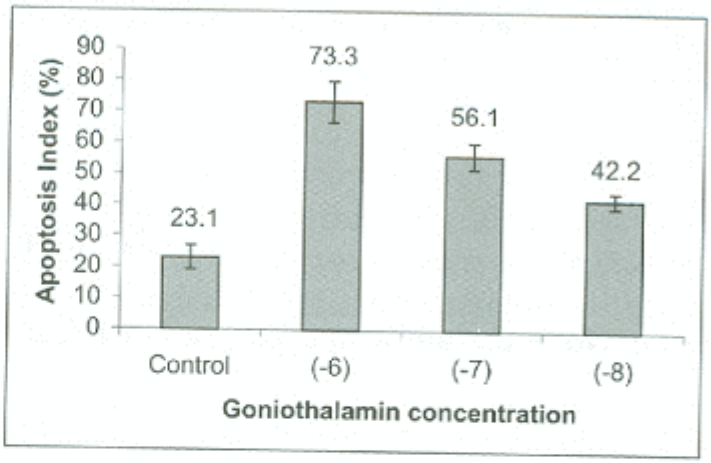

Figure 2. Apoptosis Index calculated from TUNEL Assay. Caov -3 cells were treated with different concentrations of goniothalamin (10^{-6}M, 10^{-7}M and 10^{-8}M).

Goniothalamin up-regulated Bax, but down-regulated Bcl-2

Protein expression of Bcl-2 and Bax were studied in human ovarian cancer cell line. The result from immunocytochemistry assay of Bax showed that the intensities of fluorescence in goniothalamin treated cells were increase when the concentration of goniothalamin was increased from 10^{-8}M, 10^{-7}M and 10^{-6}M, with the values of 82.9 ± 5.9, 84.3 ± 6.6 and 90.0 ± 4.0 respectively (Figure 3C). Therefore, goniothalamin was able to enhance the expression of Bax protein in this ovarian cancer cell line. Besides that, this experiment also indicated that the distribution of Bax was mainly at the cytoplasm membrane and outer nucleus membrane (Figure 3A, B), as we can see that the fluorescence was occurred at these regions.

In order to investigate the Bcl-2 protein expression in goniothalamin treated-cell line, the protein had been extracted after the immunocytochemistry study of this oncoprotein had failed to provide better picture regarding its expression. Then, SDS-PAGE electrophoresis and Western blot had been carried out. We found the presence of ~26 kDa molecule weight of Bcl-2 protein extracted from untreated cells (Figure 4). However, this band became disappeared parallel to the increase of goniothalamin treatment. This finding was very important because the over expression of Bcl-2 has been suggested that will prolong the survival of cells (Yang & Korsmeyer 1996), and can render cancerous cells relatively more resistant to the cytotoxic effect of anti-cancer treatment (Reed, 1995).

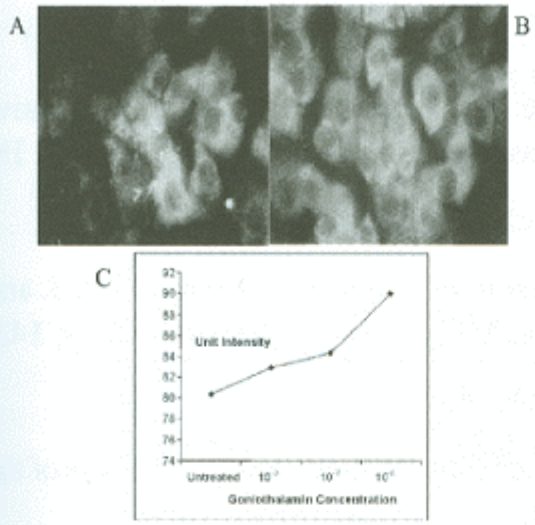

Figure 3. Expression of Bax with 10^{-6} M goniothalamin treatment (A) and untreated Caov-3 cells (B). (C) Fluorescence signal increased with goniothalamin concentration (10^{-6} M, 10^{-7} M, 10^{-8} M).

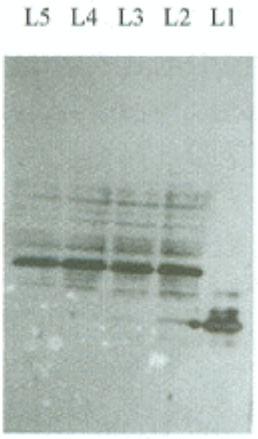

Figure 4. Western Blot of Bcl-2 protein after goniothalamin treatment (L2: untreated, L3: 10^{-6} M, L4: 10^{-7} M and L5: 10^{-8}M). L1: Standard protein of Bcl-2.

DISCUSSION

Bax and Bcl-2 are two proteins that play an important role in triggering the apoptotic pathway. Overexpression of apoptosis antagonist bcl-2 will stimulate malignant transformation and increase the resistance of cancer cells to therapeutic agents. However, Bax is the counterpart of Bcl-2 expression. Bax is reported to promote apoptosis by forming the heterodimer with Bcl-2 (Strasser *et al.*, 1997).

From the results of protein analysis, we observed that the Bax protein in gonithalamin-treated cells were increased or at least maintained at a basal level. However, Bcl-2 expression decreased significantly when the concentration of goniothalamin treatment increased. Therefore, goniothalamin may be capable to enhance the susceptibility of cancer cells towards apoptosis. By inducing the Bax production, the anti-apoptosis behavior of Bcl-2 will be neutralized by formation of heterodimer Bcl-2/Bax. When the ratio between Bax to Bcl-2, it will drive cancerous cells to undergo apoptosis.

Although most of the anticancer agents available are able to promote cell death via apoptosis, they also cause cell damage in normal tissue. Here, our results proved that plant-derived goniothalamin was able to inhibit the proliferation of Caov-3 without causing cell death in normal kidney cell as happened in tamoxifen or taxol treated cells. This was probably occurred by enhancing apoptosis pathway. Besides that, the ability of goniothalamin to increase the production of pro-apoptotic protein, Bax while decreasing the expression of Bcl-2 has made goniothalamin a potential anticancer agent in ovarian cancer therapy.

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