

Gross Thymic Extract, Thymax, Induces Apoptosis in Human Breast Cancer Cells *In Vitro* through the Mitochondrial Pathway[†]

MAMDOOH GHONEUM^{1*}, YOSHIKAZU SETO², SHIZUO SATO², ALIA GHONEUM¹,
MELISSA BRAGA¹ and SASTRY GOLLAPUDI^{3*}

¹Department of Otolaryngology, Drew University of Medicine and Science, Los Angeles, CA 90059, U.S.A.;

²Harajuku Clinic, Tokyo, Japan;

³Division of Basic and Clinical Immunology, University of California, Irvine, CA 92697, U.S.A.

Abstract. Previous studies have shown that thymic extracts possess antitumor and antimetastatic properties, but the mechanisms are not completely understood. Therefore, in this study the ability of the gross thymic extract Thymax to induce apoptosis in human breast cancer cell line (MCF-7) cells *in vitro* was evaluated. Tumor cells were cultured with different concentrations of Thymax for 24 h and the apoptotic response was assessed by propidium iodide and TUNEL assays. Activation of caspases and changes in mitochondrial membrane potential (MMP) were monitored by flow cytometry and the expression of Bcl-2 and Bax was determined by Western blot analysis. Thymax induced apoptosis in monolayer MCF-7 cells in a dose-dependent manner; at concentrations of 2.5, 5 and 10% (v/v) it caused 9%, 10% and 25% apoptosis, respectively, as compared to 6% for control cancer cells without treatment. The induction of apoptosis by Thymax was associated with activation of caspases 8 and 9, and the addition of a pan caspase inhibitor partially inhibited Thymax-induced apoptosis by 20%. In addition, the MMP was decreased significantly at Thymax concentrations of 5%-20%, which was associated with a decrease in the protein expression of Bcl-2 and an increase in Bax. These results suggest that Thymax exerts its effects via the mitochondrial pathway of apoptosis and may represent a new class of adjuvants for the treatment of breast cancer.

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*Both authors contributed equally to this work.

Correspondence to: Mamdooh Ghoneum, Ph.D., Charles R. Drew University of Medicine and Science, Department of Otolaryngology, 1621 E. 120th Street, Los Angeles, California 90059, U.S.A. Tel: +1 323 563 5953, Fax: +1 310 474 6724, e-mail: mghoneum@ucla.edu

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The thymus is the major site of production of immunocompetent T-lymphocytes from their hematopoietic stem cells. Recently, natural and synthetic thymic preparations have been used to enhance the reactivity of the host immune system to combat several diseases including cancer (1-5). Numerous thymic hormonal preparations have been used in non-specific immunological enhancement of the host anti-neoplastic response. Thymic extract causes an increase in cytolytic activity of T-cells (6), an increase in IFN γ production and improved antigen specific proliferation (7). The thymic extract, thymosin fraction 5 (TF5), a protein preparation of the bovine thymus, contains several distinct hormonal-like factors which cause an increase in the cyclic nucleotide levels of lymphocytes and lymphocyte migration and inhibitory factor production (8, 9). Earlier research also shows several of the peptide components of TF5 act on different subpopulations of T-cells (10).

Thymic extracts have been used as anticancer agents. More than twenty years of clinical studies with TF5 have shown the therapeutic potential for the treatment of patients with neoplasms (8, 11). Thymic extracts, such as prothymosin alpha 1, thymopentin and TF5, have been used to treat patients with different types of carcinomas (11-13). It has been reported that thymic hormones in combination with chemotherapeutic agents significantly increased the survival of mice with tumors (14). In addition, thymic humoral factor-gamma 2 (THF- γ 2) immunotherapy has been used as an adjunct to chemotherapeutic regimes in mice bearing immunogenic tumors (14) and in cancer patients (15-18). Previous studies have shown that thymic extracts inhibit the growth of many cancer cell lines, including neuroendocrine tumor cells and hematopoietic leukemia cell lines (19-23). For example, TF5 inhibited the proliferation of murine Dunning monocytoid leukemia cells (20, 21), L1210 murine lymphoblastic leukemia cells (22), and human acute lymphoblastic T-leukemia cells (23). The above reports suggest that thymic factors may exert their antitumor and antimetastatic effects directly by inhibiting the growth of tumor cells as well as indirectly by modulating

immune cell functions. However, the mechanisms by which thymus extracts exert a direct anticancer activity are not completely understood. Therefore, in this study the ability of Thymax, a gross extract of thymus, to induce apoptosis in human breast cancer cell line (MCF-7) cells *in vitro* and the possible mechanisms underlying its effect were examined.

Materials and Methods

Tumor cell line. A human breast cancer cell line (MCF-7) was used in the current study. The cell line was purchased from the American Type Tissue Collection, Manassas VA, USA.

Complete medium (CM). CM consisted of RPMI-1640 supplemented with 10% fetal calf serum and 1% antibiotics (100 U penicillin and 100 µg/ml streptomycin).

Thymax. Thymax is a thymus gross extract with heat-resistant peptides contained in acidic pH (through treatment with NaCl and L-ascorbic acid). The thymus is obtained from a pathogen-free pig. The thymus is cut into pieces, heated rapidly to decrease its protease activity, purified water is added to homogenize, and the extract is then stirred at 300 rpm for one minute. The cell homogenate is heated again at 80°C for 30 minutes, cooled down to 37°C and then filtered to obtain several peptide fractions and free amino acids (high performance liquid chromatography, HPLC analysis). Finally, NaCl and L-ascorbic acid are added. Thymax was offered by YS Nature Company, Tokyo, Japan.

Thymax differs from other thymus extracts in that it is a complex of thymosin, thymomodulin and many other peptides. In addition, unlike other thymic factors, Thymax is introduced into the body by mouth. Attempts are currently being made to identify the active factors.

Apoptosis studies.

(i) Detection of cancer cell viability using propidium iodide. The MCF-7 cells were cultured in the presence or absence of Thymax (1.25-10%) for 3 days and the percentage of dead cancer cells was examined by the propidium iodide (PI) technique using a FACScan flow cytometry. In this technique, dead cells pick up PI and fluoresce. Briefly, PI was added to the cells (1×10^6 /ml) to give a final PI concentration of (50 µg/ml). The cells were stained for 30 minutes at room temperature in the dark and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA).

(ii) Detection of apoptotic cancer cells by TUNEL assay. DNA strand breaks were measured by the terminal deoxynucleotidyl transferase (TdT) dUTP-mediated nick end labeling (TUNEL) assay. The cells (2×10^5 cells/ml) were incubated at 37°C for 48 h with or without Thymax. The cells were then fixed with 2% formaldehyde for 15 minutes at room temperature and then washed with PBS and permeabilized with 2% sodium citrate and 10% Triton X-100 for 5 minutes on ice. After washing, the cells were incubated with FITC-dUTP in the presence of the TdT enzyme solution containing 1 µM potassium cacodylate and 125 µM Tris-HCL (pH-6.6, *In Situ* Detection Kit, Boehringer Mannheim, Indianapolis, IN, USA) for 1hr at 37°C. After incubation, the cells were washed with PBS, and 10,000 cells were acquired and analyzed using FACScan and Cell Quest software (Becton Dickinson, Menlo Park, CA, USA).

(iii) Determination of activation of caspases 8 and 9. In order to determine the steps in Thymax-mediated apoptosis, the activation of proximal caspases (caspase-8 and caspase-9) was examined. The MCF-7 cells were treated with Thymax for 48 h and the proportion of cells with active caspase 8 and caspase 9 were determined with a caspase detection kit using FACScan. The method is based on carboxyfluorescein-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-toxic. Once inside the cells, these inhibitors bind covalently to the active caspase. Caspase positive (+) cells are distinguished from caspase negative (-) cells with the aid of flow cytometry. Briefly, cells undergoing apoptosis were loaded with fluorescein-labeled FMK-peptide inhibitors (FAM-LETD-FMK [a carboxyfluorescein derivative of benzyloxycarbonyl leucylglutamylthreonylaspartic acid fluoromethyl ketone (zLETD-FMK)] for caspase-8, FAM-LEHD-FMK [a carboxyfluorescein derivative of benzyloxycarbonyl leucylglutamylhistidylaspartic acid fluoromethyl ketone (zLEHD-FMK)] for caspase-9, Cell Technology Inc, Mountain View, CA, USA). After 1 h incubation, the cells were washed to remove unbound caspases, and the cells that contained bound inhibitor were quantified using a FACScan flow cytometer.

(iv) Caspase inhibitor. To study the role of caspases in apoptosis, 1×10^6 MCF-7 cells were cultured with or without Z-VAD-FMK (Z-Val-Ala-DL-Asp-fluoromethylketone, broad-spectrum caspase inhibitor, BioVision, Palo Alto, CA, USA), for 2 h and were subsequently cultured in the presence or absence of Thymax. The percentage of dead cancer cells was examined by flow cytometry as described above.

(v) Detection of mitochondrial membrane potential (MMP). Variations of the mitochondrial transmembrane potential $\Delta\Psi_m$ during apoptosis were studied using tetramethylrhodamine ethyl ester, (TMRE, Molecular Probes, Eugene, OR, USA). Briefly, after treatment with Thymax for 3 days 5×10^5 cells/ml were incubated with 50 nM TMRE for 30 minutes at 37°C. The cells were washed with PBS and analyzed with FACS Forward, the side scatters were used to gate and exclude cellular debris using a FACScan. The cells were excited at 488 nm and the emission was collected on the FL2 channel. Five thousand cells were analyzed. The data were acquired and analyzed using Cell Quest software (Becton-Dickinson).

(vi) Western blot. The expression of Bax and Bcl-2 was determined using Western blot analysis. The cells were cultured with Thymax at different concentrations (5, 10, and 20% v/v) for 3 days. The cells were harvested, washed with cold PBS (10 mmol/L [pH 7.4]), and lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L ethylene glycol tetraacetic acid [EGTA], 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4 , 1% NP40, 1 mmol/L-phenylmethylsulfonyl fluoride, 10 Ag/mL aprotinin, and 10 Ag/mL leupeptin [pH 7.4]) for 30 minutes and centrifuged at $14,000 \times g$ for 20 minutes at 4°C as detailed previously. The supernatant was collected and used immediately. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Seventy-five µg of the cell lysate in lysis buffer were subjected to Western blot analysis by 4%-12% SDS polyacrylamide gel electrophoresis using 1:500 anti-Bax antibody or anti-Bcl-2 antibody (BD Biosciences, San Jose, CA, USA) as primary antibody. The washed polyvinylidene fluoride (PVDF) membranes were incubated with a 1:2000 dilution of monoclonal secondary antibodies. The immunoreactive bands were visualized by using an ECL Western

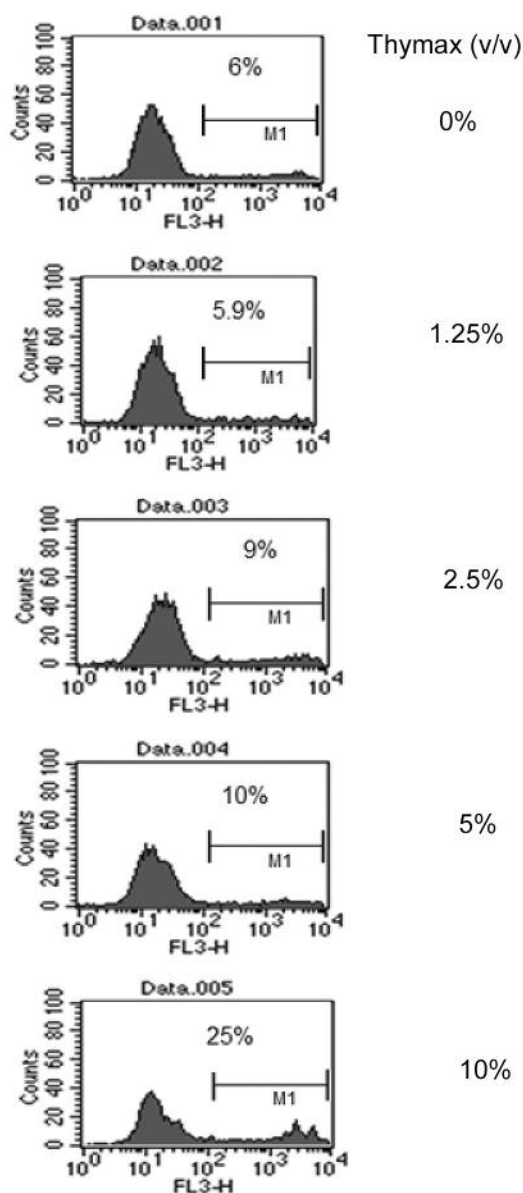


Figure 1. Percentage of dead cancer cells post treatment with Thymax as determined by propidium iodide (PI) followed by flow cytometry. MCF-7 cells were cultured in the presence or absence of different concentrations of Thymax (5-20%) for 3 days, and cancer cell survival was determined by PI staining followed by flow cytometry. Numbers on the graphs represent the percent apoptosis.

Blotting Kit (Amersham, Buckinghamshire, UK). To verify equal protein loading and transfer, the blots were stripped and re-probed with β -actin using an anti-actin rabbit polyclonal antibody; thereafter the same protocol was followed as detailed above. The relative intensity of each protein band in a blot was measured by using the computerized software program OPTIMAS 6.2 (Media Cybernetics, Silver Spring, MD, USA) and the ratio of Bcl-2 to Bax was analyzed (24). The band intensities were determined using Quantity One software from Bio-Rad. The data are represented as mean \pm SEM.

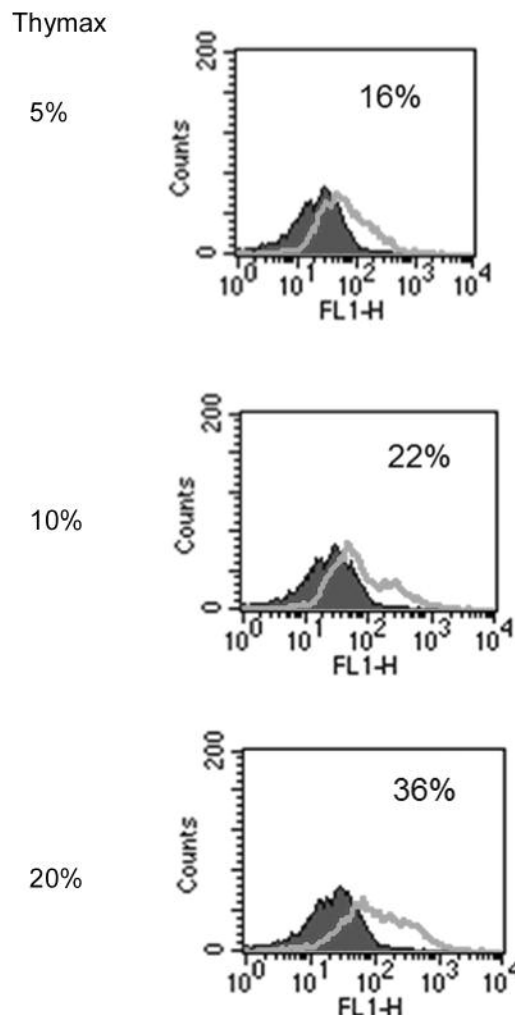


Figure 2. Percentage of dead cancer cells post treatment with Thymax as determined by TUNEL assay. MCF-7 cells were cultured in the absence or in the presence of Thymax at different concentrations for 48h and cancer cell survival was determined by TUNEL assay. Numbers on the graphs represent the percent apoptosis.

Statistical analysis. The differences in the Bax and Bcl-2 ratios between Thymax treated and untreated cells were analyzed by ANOVA. All the comparisons were two-tailed and p -values less than 0.05 were considered statistically significant. The Student's t -test was performed to determine the statistical significance of the difference in the absolute values of the apoptotic cell death between the Thymax-treated and non-treated groups. The Thymax-induced apoptosis was considered significant at $p < 0.05$ unless otherwise specified.

Results

Effect of Thymax on tumor cell survival.

(i) *Flow cytometry.* The data in Figure 1 demonstrates that Thymax in a dose-dependent manner increased apoptosis in the MCF-7 cells. The apoptotic effect of Thymax was detected at

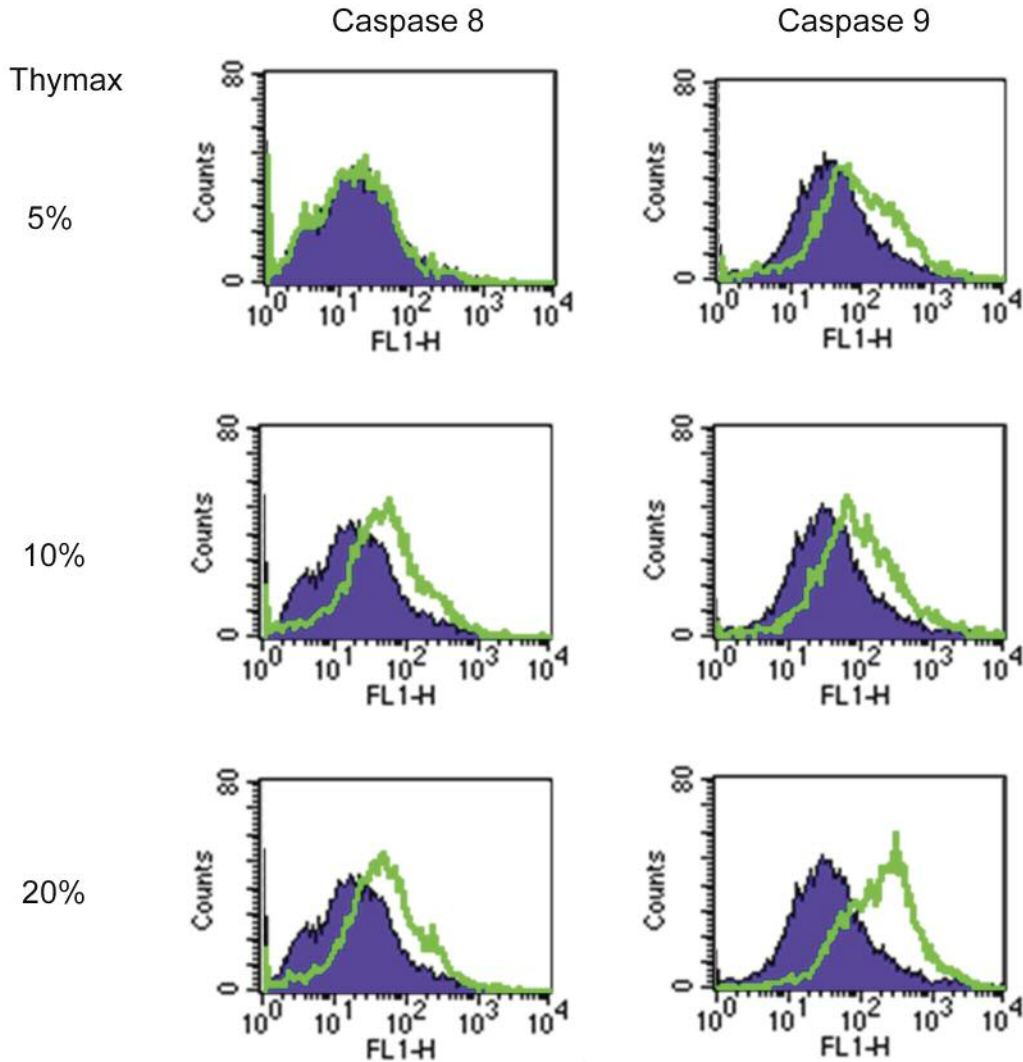


Figure 3. Increased activation of caspases 8 and 9 of MCF-7 cells post culture with Thymax. MCF-7 cells were incubated with Thymax and intracellular active caspases-8 and caspase-9 were determined with casp glow caspases 8 and 9 determination kit using FACScan. Representative dot blots showing increased activation of caspases, which was highest at 20% Thymax for caspase-9. The blue area represents the control, and the green line represents cells treated with Thymax.

concentrations as low as 2.5% (9% apoptosis) and was highest at a concentration of 10% (25% apoptosis), as compared to the control, untreated MCF-7 cells (6% apoptosis).

(ii) *TUNEL assay.* Figure 2 shows that Thymax in a dose-dependent manner induced apoptosis in the MCF-7 cells and this effect was highest at a concentration of 20% (36% apoptosis), as compared to the control, untreated MCF-7 cells (5% , data not shown).

(iii) *Effect of Thymax on activation of caspases 8 and 9.* Figure 3 shows representative histogram plots for the activation of caspases. Thymax induced activation of caspase-8 and caspase-9 in a dose-dependent manner. However, the

activation of caspase-9 was more remarkable, it was detected at a concentration of Thymax as low as 5% and was highest at a concentration of 20% .

(iv) *Apoptosis in the presence of caspase inhibitor.* Figure 4 shows that treatment of the MCF-7 cells with Z-VAD-FMK, a caspase family inhibitor, partially inhibited Thymax-induced apoptosis in the MCF-7 cells (20% reduction in apoptosis) as compared with the control cells treated with Thymax alone.

(v) *Mitochondrial membrane potential.* Figure 5 shows that the Thymax-treated MCF-7 cells revealed a significant decrease in the mitochondrial polarization which was detected at concentrations of 5% and maintained at 20% .

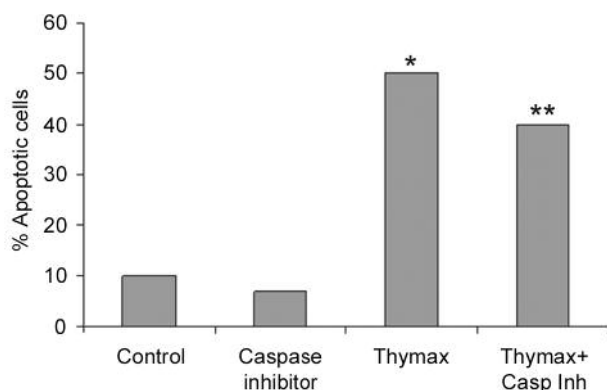


Figure 4. Apoptosis of MCF-7 cells in the presence of caspase inhibitors. MCF-7 cells were cultured with or without Thymax in the presence or absence of Z-VAD-FMK (caspase family inhibitor) for 3 days and apoptosis was determined by PI technique. * $p < 0.001$ as compared to control untreated cells. ** $p < 0.02$ as compared to Thymax-treated cells.

(vi) Effect of Thymax on protein expressions of Bcl-2 and Bax. Figure 6 depicts the results of Western blot analysis showing that treatment of cancer cells with Thymax for 3 days resulted in significant down-regulation of the antiapoptotic protein Bcl-2 ($p < 0.001$) concomitant with up-regulation of Bax ($p < 0.01$) as compared to control untreated cells.

Discussion

Thymax induced apoptosis in the human breast cancer cells. The induction of apoptosis was associated with disruption of the MMP, activation of caspase-9 and a decrease in the Bax:Bcl-2 ratio. Recently, Spangelo *et al.* (25) have shown that decreased proliferation of HL-60 cells treated with TF5 was associated with apoptosis, as indicated by an increased subdiploid fraction (*i.e.*, sub-G1) and Annexin V staining. In the present study, we have shown that thymic extracts have the ability to induce apoptosis in another type of cancer, human breast cancer, by a mechanism that involves MMP.

Apoptosis, or programmed cell death, is a distinct form of cell death controlled by an internally encoded suicide program (26-28). The two most common apoptotic pathways are the extrinsic pathway and the intrinsic pathway (mitochondrial pathway) (28-30). The extrinsic pathway is initiated by the stimulation of death receptors (such as CD95, TNFR 1) in the plasma membrane (30, 31). The intrinsic pathway is initiated by chemotherapeutic agents, radiation and other cellular stresses that disrupt mitochondrial integrity (28, 32, 33). Mitochondria play an important role in the intrinsic pathway of apoptosis which proceeds via the pathway involving MMP (28). Mitochondria contain two well-defined compartments: the matrix, surrounded by the inner membrane (IM) and the intermembrane space, surrounded by the outer membrane

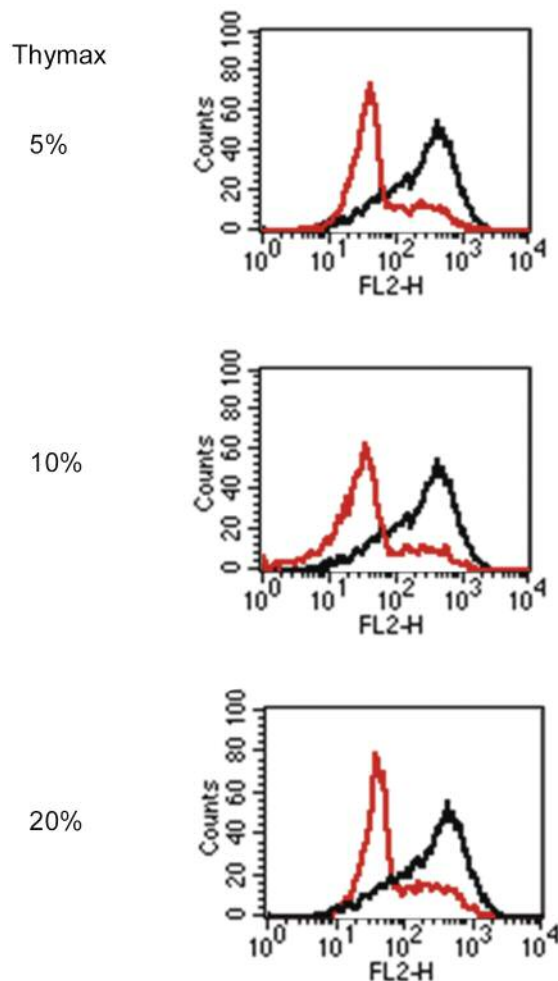


Figure 5. Effect of Thymax on mitochondrial membrane potential. MCF-7 cells were cultured with Thymax for 3 days and the mitochondrial membrane potential was determined by FACScan using FACS Forward. The black lines represent control cells, and the red lines represent cells treated with Thymax. Depolarization is indicated by decreased fluorescence.

(OM). The IM contains various molecules, including ATP synthase, the electron transport chain, and adenine nucleotide translocator. Under physiological conditions these molecules allow the respiratory chain to create an electrochemical gradient or MMP. IM permeabilization leads to changes in MMP (34, 35). Bcl-2 is located on the IM and appears to play an important role in the maintenance of MMP. The intermembrane space contains cytochrome c, certain pro-caspases, and the apoptosis-inducing factor (AIF). The permeabilization of the mitochondrial membranes results in the release of proapoptotic molecules into the cytosol. The release of cytochrome c triggers the assembly of Apaf-1 (apoptotic protease-activating factor) and pro-caspase-9 to form an apoptosome (28, 34). Pro-caspase-9 is dimerized and activated, and active caspase 9 activates executioner caspases

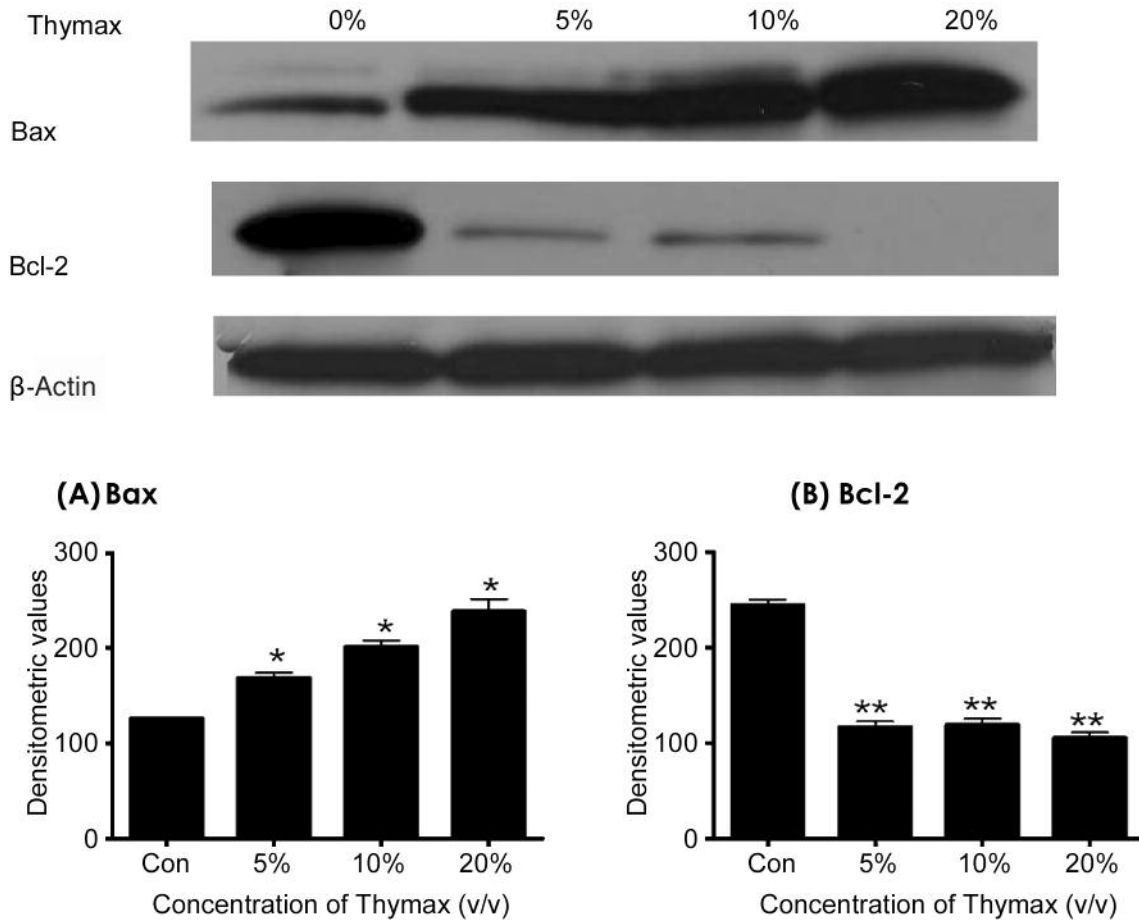


Figure 6. Western blot analysis of the effect of Thymax on the expression of proapoptotic protein Bax (A) and (B) antiapoptotic protein Bcl-2 in MCF-7 cells. The ratio of Bax and Bcl-2 proteins expression was determined by comparing the relative intensities of protein bands. Data are represented as mean±SEM from 3 separate experiments. MCF-7 cell lysates were prepared and the expressions of Bax and Bcl-2 proteins were examined as described in Materials and Methods. Representative blot from 3 separate experiments with identical results. * $p < 0.01$, ** $p < 0.001$ as compared to control untreated MCF-7 cells.

in order to orchestrate apoptosis. AIF induces apoptosis by directly bypassing activation of the caspases. In the present study, Thymax activated caspase 8 and to a greater extent caspase 9. However, the caspase family inhibitor partially inhibited the Thymax-induced apoptosis, suggesting that both caspase dependent and independent pathways play a role in Thymax-induced apoptosis.

The mitochondrial pathway is mainly governed by the Bcl-2 family of proteins, which include pro-apoptotic (Bax, Bad, Bid, *etc.*) and anti-apoptotic (Bcl-2, Bcl-XL) proteins. The ratio of pro-apoptotic proteins (such as Bax) *versus* anti-apoptotic proteins (such as Bcl-2), determines the sensitivity or resistance of cells to various apoptotic stimuli (36). The present results indicated that Bax was upregulated and Bcl-2 was down-regulated in the Thymax-treated MCF-7 cells. Therefore, the increased Bax:Bcl-2 ratio may play a critical role in the apoptotic activity of Thymax.

Conclusion

Thymax exerts its effects *via* the mitochondrial pathway of apoptosis and may represent a new class of adjuvants for the treatment of breast cancer.

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