

Wortmannin induces MCF-7 breast cancer cell death via the apoptotic pathway, involving chromatin condensation, generation of reactive oxygen species, and membrane blebbing

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Background: Apoptosis can be used as a reliable marker for evaluating potential chemotherapeutic agents. Because wortmannin is a microbial steroidal metabolite, it specifically inhibits the phosphatidyl inositol 3-kinase pathway, and could be used as a promising apoptosis-based therapeutic agent in the treatment of cancer. The objective of this study was to investigate the biomolecular mechanisms involved in wortmannin-induced cell death of breast cancer-derived MCF-7 cells.

Methods and results: Our experimental results demonstrate that wortmannin has strong apoptotic effects through a combination of different actions, including reduction of cell viability in a dose-dependent manner, inhibition of proliferation, and enhanced generation of intracellular reactive oxygen species.

Conclusion: Our findings suggest that wortmannin induces MCF-7 cell death via a programmed pathway showing chromatin condensation, nuclear fragmentation, reactive oxygen species, and membrane blebbing, which are characteristics typical of apoptosis.

Keywords: wortmannin, human breast adenocarcinoma, apoptosis, reactive oxygen species, flow cytometry

Introduction

Breast cancer is the most common human malignancy and causes considerable cancer-related mortality in the Western world, where approximately one-third of women develop metastases.¹⁻⁵ Systemic breast cancer therapies have limitations in terms of nonspecific targeting, toxicity to normal tissues, and increased drug resistance, resulting in only short-term efficacy.^{2,6} Using conventional therapy, 5-year and 10-year survival rates for locally advanced breast cancer are approximately 55% and 35%, respectively.^{7,8} However, 10 years after diagnosis and removal of the primary tumor, a 40% recurrence rate has been reported.^{8,9} Therefore, novel and advanced alternative therapeutic strategies are needed.

Apoptosis is a programmed cell death process and a natural phenomenon that is important in both normal physiological and pathological conditions.² An ideal anticancer drug would inactivate cancer cells without much more effect in normal cells.¹⁰ By inducing apoptosis specifically in cancer cells, this ultimate goal can be achievable. Phosphatidyl inositol 3-kinase (PI3K) helps cancer cells to avoid apoptosis and promotes cell cycle progression and proliferation as well as angiogenesis by modulating proapoptotic molecules, such as Bad and p53,¹¹⁻¹⁵ enabling cancer cells

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to grow in an uncontrolled manner. Furthermore, mutated PI3K activation is abnormally expressed or overexpressed in several cancers, including stomach, colon, breast, lung, ovarian, and pancreatic cancer, with activation of its main downstream gene, Akt.^{11,16,17} A study has concluded that PI3K-Akt plays a major role in this cell line with regard to increased drug resistance.^{18,19} Deregulated PI3K-Akt activity has also been reported in breast malignancies associated with increased resistance to multiple chemotherapeutics and radiotherapies.^{18,20–22}

The MCF-7 cell line has been widely used as a model breast cancer cell line due to its inherent lack of functional caspase 3.^{23,24} Because MCF-7 cells lack this pivotal crucial effector protease, they become highly resistant to most chemotherapeutic drugs and subsequently survive because of resistance to apoptosis,^{1,25} and do not undergo classical apoptogenic mechanisms during programmed cell death.²⁶ Studies that have used the MCF-7 cell line with conventional breast cancer chemotherapeutic agents, such as paclitaxel, doxorubicin, 5-fluorouracil, etoposide, and camptothecin, have reported resistance within a few months to a few years.^{18,19} Therefore, the PI3K-Akt pathway is of great interest as a novel target in the treatment of breast cancer.

Wortmannin (C₂₃H₂₄O₈, Figure 1) is a highly cell-permeable, antifungal-antibiotic agent similar to the viridian group, and a potent and irreversible PI3K inhibitor that blocks the PI3K-Akt signaling pathway involving cell cycle progression and apoptosis.^{12,17} Therefore, we hypothesized that wortmannin could induce apoptosis in MCF-7 breast cancer cells. Various modifications and conjugations with wortmannin have been reported, establishing it as an effective antitumor agent.^{16,27} However, in the present study, the direct apoptotic effect of wortmannin at various concentrations was investigated selectively in the MCF-7 cell line. Thus, the primary goal of these experiments was

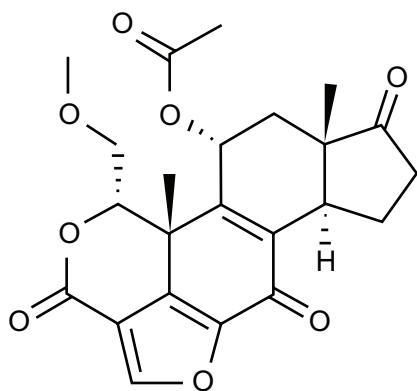


Figure 1 Chemical structure of wortmannin.

to determine the apoptotic effects of wortmannin in a widely used MCF-7 cell model.

Materials and methods

A MCF-7 breast cancer cell line, Eagle's Minimum Essential Medium (EMEM), bovine insulin, penicillin-streptomycin, and trypsin-ethylenediamine tetra-acetic acid (TE) was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). Trypan blue, Hoechst 33342, acridine orange, ethidium bromide, propidium iodide, H₂O₂, and phosphate-buffered saline (PBS) was sourced from Amresco Inc (Salon, OH). 2', 7'-Dichlorofluorescein diacetate (DCF-DA), and Yo-PRO[®]-1 and propidium iodide (PI) double staining kits were purchased from EMD Chemicals (Gibbstown, NJ) and Invitrogen (Life Technologies, Carlsbad, CA), respectively. MTT [3-(4, 5-dimethylthiazol2-yl) – 2.5-diphenyltetrazolium bromide], dimethyl sulfoxide, fetal bovine serum, and a standard tissue culture (75 cm²) flask with a filtered cap was purchased from BioExpress (Kaysville, UT).

MCF-7 cell culture

MCF-7 cells were obtained from the ATCC and maintained in EMEM 10% fetal bovine serum, 0.01 mg/mL bovine insulin, 100 U/mL of penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Cells were grown in a standard tissue culture (75 cm²) flask with a filtered cap, and the growth media were changed every 2–3 days as per the ATCC protocol. Cells were subcultured with 0.25% TE after reaching 70% confluence. For all experiments, the cells were seeded on 6-well, 12-well, and 96-well plates at a density of 5 × 10⁴ cells/well, 2.5 × 10⁴ cells/well, and 1 × 10⁴ cells/well, respectively, and allowed to attach before applying various treatments.

Viability, cell death, and cytotoxicity using Trypan blue exclusion assay

The Trypan blue exclusion method was used with some modifications to determine total cell numbers and the proportion of live and dead cells. Briefly, MCF-7 cells were grown on 6-well plates at a density of 5 × 10⁴ cells/well. Replacement of starvation media was performed as usual at 24 hours before treatment with wortmannin. The cells were treated with different concentrations of wortmannin for 24 hours and then trypsinized, centrifuged, redispersed in 0.5 mL of media, mixed thoroughly with 50 µL of 0.4% Trypan blue (w/v), and allowed to stain at room temperature for 5 minutes. The cells were then counted using a hemocytometer. Each experiment was repeated three times with triplicate samples. In statistical

analysis, the percentage of cell death was calculated by counting at least 200–300 cells per sample in several randomly selected fields using the formula of percentage of cell death = number of dead cells/number of total cells \times 100.

MTT assay

Cell metabolic activity was determined using a modified MTT assay. Briefly, MCF-7 cells were seeded at a density of 1.25×10^4 cells/well in 24-well plates. EMEM was replaced by 0.5 mL of starvation medium on the day before the experiments. Different concentrations of wortmannin were then applied, with incubation at 37°C in 5% CO₂ for 24 hours. The cells were then labeled with 20 μ L of MTT solution (5 mg/mL in PBS) added into each well, followed by 5 minutes of orbital shaking, after which the cells were incubated in the dark for 4 hours. The medium was then removed, dimethyl sulfoxide (DMSO) was added to dissolve the formazan, and photographs were taken using a phase-contrast microscope. Three separate experiments were conducted.

Phase-contrast microscopy for morphological analysis

MCF-7 cells were seeded in triplicate into 6-well plates at a density of approximately 5×10^4 cells/well and treated with various concentrations of wortmannin. For qualitative assessment, images were taken randomly using a Zeiss Axiovert 40 CFL inverted phase-contrast microscope (Zeiss, Oberkochen, Germany).

Fluorescence microscopy for analysis of apoptosis

Apoptotic cell death was quantified using acridine orange and ethidium bromide as fluorescent probes. Briefly, cells treated or not treated with wortmannin were trypsinized, centrifuged, pelleted, and stained with 20 μ L of dye mixture containing 15 mg/mL of acridine orange and 50 mg/mL of ethidium bromide in 1 \times PBS and allowed to stain for 5–10 minutes at room temperature. Thereafter, 2–3 drops of the stained cells were placed on each slide with a coverslip. Photographs were taken at random locations under a Nikon 90i fluorescence microscope equipped with Nikon BR software. Untreated cells were used as the negative control.

Flow cytometric apoptosis assay using Yo-PRO®-I and propidium iodide staining

Apoptosis was quantified by Yo-PRO®-I and propidium iodide double-staining methods using a vibrant apoptosis detection kit. Briefly, the cells were treated with different

concentrations of wortmannin for 24 hours. The cells were then collected by trypsinization, and resuspended in ice-cold 1 \times PBS at a density of about 1×10^6 cells/mL in PBS. The cells were double-stained with Yo-PRO®-I and propidium iodide according to each manufacturer's protocol, followed by 30 minutes of incubation on ice. The cells were then analyzed by BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) using CellQuest® Pro software.

Nuclear chromatin staining with Hoechst 33342

MCF-7 cells (about 5×10^4 cells/well) were plated on coverslips in 6-well plates and treated with different concentration of wortmannin for 24 hours. After incubation, the cells were stained with 1 μ g/mL Hoechst 33342 dye (diluted with distilled water) for 20 minutes in a 37°C incubator with 5% CO₂. The cells on the coverslips were then washed in ice-cold PBS (1 \times) three times, after which the cells were mounted on glass slides and photographed immediately using a 90i Nikon fluorescent microscope.

Measurement of intracellular ROS

Cells treated with wortmannin were grown on 6-well plates as described in the cell culture section for measurement of intracellular ROS accumulation and quantification. After 24 hours of incubation, the cells were stained with 5 μ M ROS-specific H₂O₂-sensitive DCF-DA dye and incubated at 37°C for 30 minutes. The cells were then trypsinized, pelleted, and washed three times with ice-cold PBS. Negative control cells are not treated with wortmannin. As a positive control, H₂O₂ (0.3%) treated cells were used.²⁸ The cells were then analyzed for fluorescence intensity using the FACSCalibur flow cytometer and CellQuest Pro software.

Statistical analysis

Experiments were done in triplicate. The data are expressed as the mean \pm standard error. Significance was evaluated by one-way analysis of variance and the Student's *t*-test.

Results

Effects of wortmannin on MCF-7 cell growth, toxicity, and viability

The Trypan blue exclusion assay was used to determine the amount of MCF-7 cell death. MCF-7 cells were incubated with 0–500 nM of wortmannin for 24 hours to assess their cellular growth and toxicity using the Trypan blue exclusion assay. As shown in Figure 2, wortmannin induced cell death in MCF-7 cells compared with untreated controls in a

concentration-dependent manner as determined by Trypan blue staining. The 50% inhibitory concentration (IC_{50}) of wortmannin was calculated to be 400 nM over 24 hours (Figure 2).

Effect of wortmannin on cellular morphology

As shown in Figure 3, distinctive morphological changes were observed at higher concentrations and over a longer time period compared with controls, indicating induction of apoptosis in a dose-dependent manner. Thus, the phase-contrast micrographs of wortmannin-treated MCF-7 cells show typical apoptotic morphological characteristics.

Biochemical and morphological analysis of apoptosis

To confirm whether the reduced cell viability was due to apoptosis, acridine orange and ethidium bromide fluorescence staining was used to detect apoptotic cells. Using fluorescence microscopy, uniform normal green cells were observed in the control group (Figure 4A), whereas an increased number of dark orange or red cells with increased wortmannin concentrations were detected (Figure 4B–D). Results are from three independent experiments.

Qualitative MTT assay

Reduced cell viability was qualitatively determined by MTT assay after the cells were treated with various concentrations of wortmannin, as detected by phase-contrast microscopy (see Figure 5). The morphological features of wortmannin-treated MCF-7 cells demonstrated reduced purple formazan growth. Wortmannin inhibited proliferation of MCF-7 cells in a dose-dependent

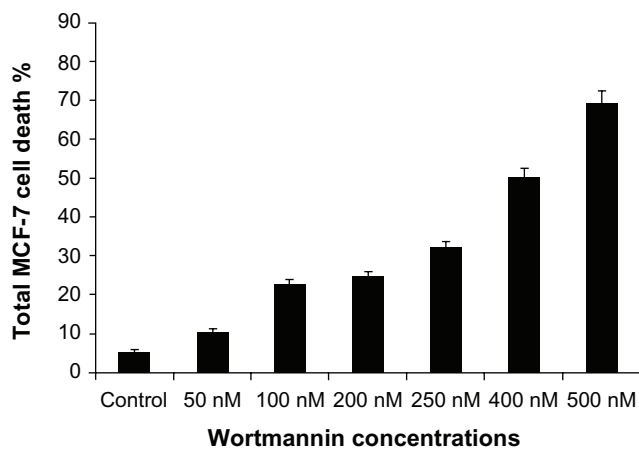


Figure 2 Cell death determination by Trypan blue exclusion assay.

Notes: Cells were treated with wortmannin and incubated for 24 hours. Results are shown as the mean \pm standard error, for at least three individual experiments, in triplicate.

manner. The results indicated that cytotoxicity of wortmannin in cancer cells was significant when compared with the control. The cytotoxic MTT assay result shows dose-dependent death of MCF-7 cells with wortmannin treatment.

Wortmannin quantitatively induces apoptosis in MCF-7 cells

To elucidate further the mechanisms of cell death in wortmannin-induced MCF-7 cells, we analyzed the quantitative flow cytometry data from FACSCaliber using Yo-PRO[®]-1 and propidium iodide double-staining. The bottom right quadrant (LR) defined the percentage of early apoptotic cells with Yo-PRO[®]-1 staining; the top right quadrant (UR) defined the percentage of late apoptotic cells (Yo-PRO[®]-1 and propidium iodide-stained cells). In the quadrant histogram shown in Figure 6, Yo-PRO[®]-1-positive cells and propidium iodide-negative cells in the LR quadrant were considered to be early apoptotic cells, and Yo-PRO[®]-1-positive and propidium iodide-positive cells in the UR quadrant were considered to be late apoptotic cells. MCF-7 cells treated with wortmannin for a 24-hour period are shown in Figure 6. The percentage of cells in the early stages of apoptosis gradually increased, ranging from 4.44% to 36.38% in a dose-dependent manner compared with the control group (0.21%). Furthermore, the total percentage of cells (early and late apoptotic) also increased from 63.82% to 74.42% in a dose-dependent manner compared with the control group (8.41%).

Nuclear chromatin condensation with Hoechst 33342

The effect of wortmannin on nuclear morphology using fluorescence microscopy is demonstrated in Figure 7. We observed that the nuclei of wortmannin-treated cells showed hypercondensed chromatin strongly bound to fluorescent dyes, with a brighter appearance along with shrunken and fragmented nuclei. This allowed clear discrimination from untreated nonapoptotic MCF-7 cells with smooth, flattened nuclear morphology and normal nuclei, as well as uniformly dispersed chromatin. Thus, wortmannin-treated breast cancer MCF-7 cells demonstrated a chromatin condensation effect and confirmed that the mechanism of cell death was via apoptosis.

Microscopic apoptotic blebbing

To study the mechanisms of cell death, we investigated whether wortmannin induces programmed cell death through the apoptotic pathway. Phase-contrast microscopy was used after treatment with wortmannin for 24 hours. Morphological apoptotic features, such as cell shrinkage, membrane blebbing,

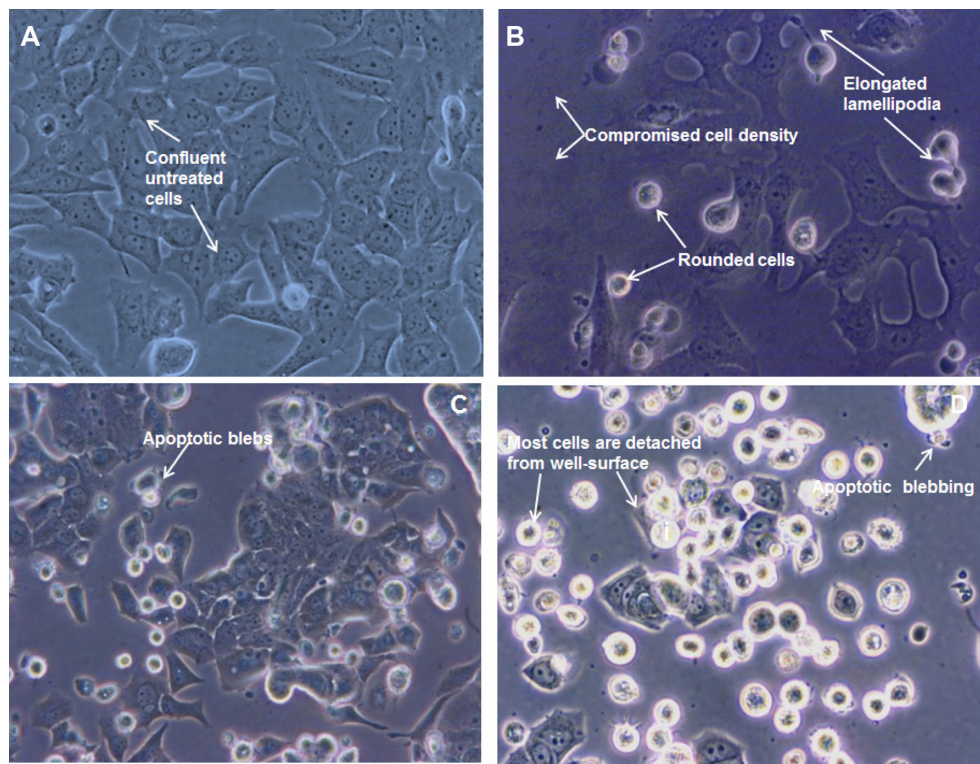


Figure 3 Phase-contrast micrographs of wortmannin treated MCF-7 cells incubated for 24 hours.

Notes: (A) Control cells show dense cell populations with very few rounded cells. (B) At low concentrations (100 nM), wortmannin-treated cells reveal increased numbers of rounded cells with compromised cell density. (C) At 500 nM, wortmannin-treated cells show more rounded cells with fewer viable cells. (D) At 2 μ M, most of the wortmannin-treated cells have a rounded, shrunken, and suspended appearance with apoptotic blebbing.

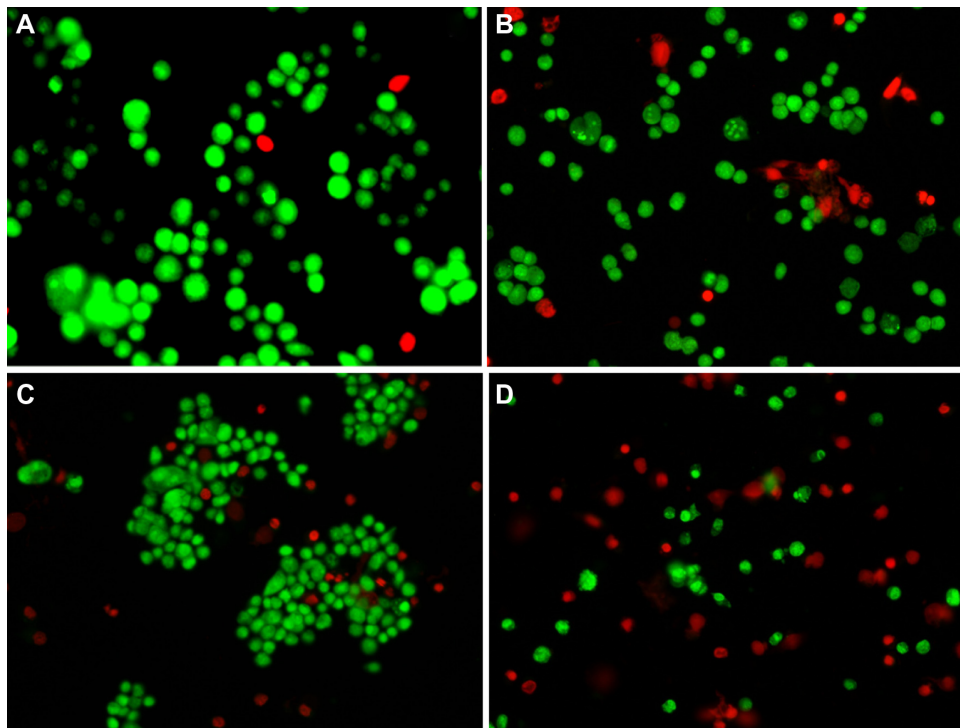


Figure 4 Fluorescence micrographs of MCF-7 cells treated with different concentrations of wortmannin and stained with acridine orange and ethidium bromide. Viable cells show green fluorescence and apoptotic cells show dark orange or red fluorescence. (A) Control cells not treated with wortmannin, and treated with wortmannin (B) 500 nM, (C) 1 μ M, and (D) 5 μ M for 24 hours.

Note: Representative photographs indicate that wortmannin-treated MCF-7 cells are undergoing programmed cell death.

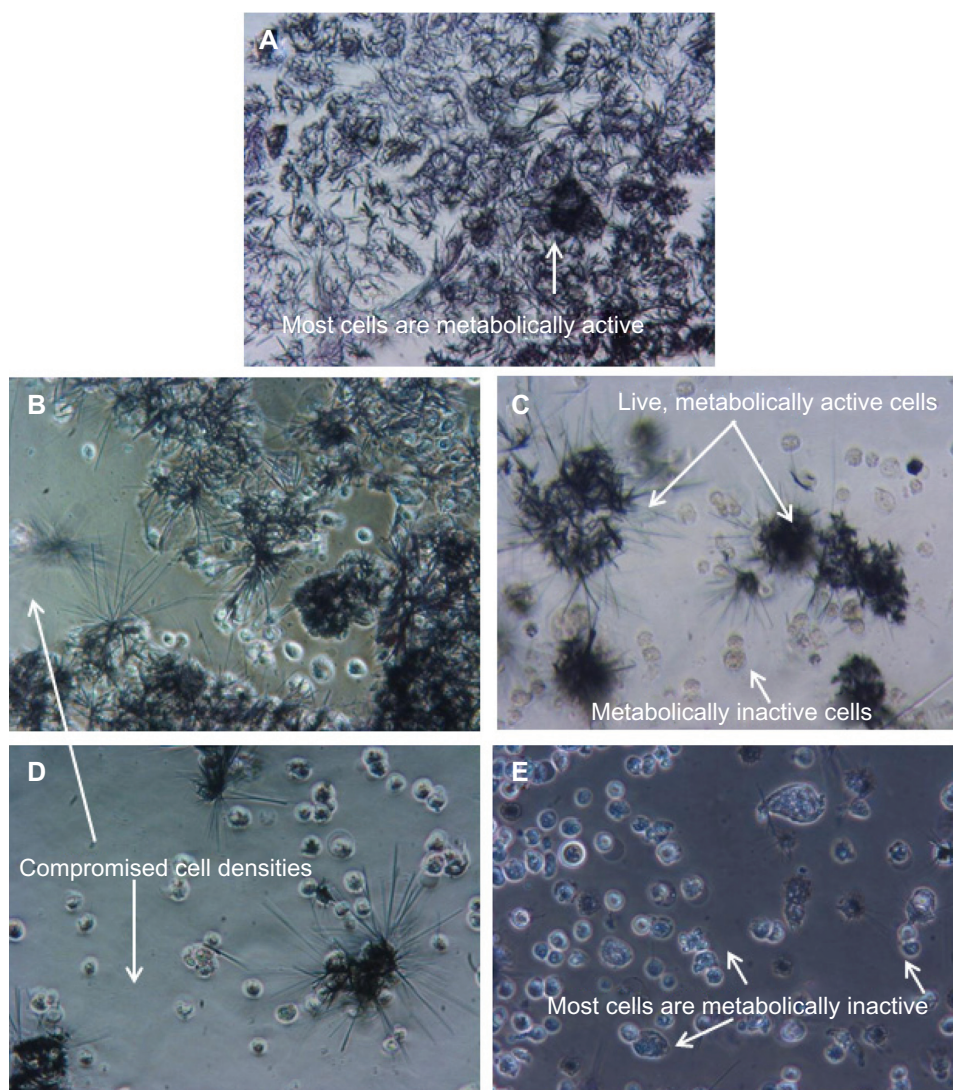


Figure 5 Representative phase-contrast micrographs of qualitative MTT assay. (A) Control cells and those treated with wortmannin (B) 500 nM, (C) 1 μ M, (D) 2 μ M, and (E) 4 μ M, for 24 hours.

rounding up of cells, and a decrease in volume were used as standard apoptosis markers, as shown in Figure 8.^{2,10,25}

Increased intracellular ROS levels in wortmannin-treated MCF-7 cells

Next we evaluated whether wortmannin-induced apoptotic MCF-7 cells trigger signaling responses via intracellular generation of ROS. As shown in Figure 9, the level of ROS generation was significantly increased compared with controls in wortmannin-treated MCF-7 cells using the ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate probe through FACS analysis. Corresponding geometric means of total fluorescence intensity was calculated for different levels of fluorescence.^{29–31} Wortmannin treatment increases intracellular ROS accumulation in MCF-7 cell lines. This

result suggests that an increase in total cellular ROS generation triggers wortmannin-induced programmed cell death in MCF-7 cells via apoptotic pathways.^{1,2,28–33}

Discussion

Most breast cancer cells become resistant to current chemotherapeutic drugs due to mutation of apoptotic mechanisms.³⁴ It has been reported that MCF-7 cells are resistant to most of the drugs approved by the US Food and Drug Administration, including paclitaxel, doxorubicin, 5-fluorouracil, etoposide, and camptothecin, with the conclusion that the PI3k-Akt pathway plays a major role in increased resistance.¹⁸ In addition, another study revealed that the Akt level was higher in MCF-7 cells than in other breast cancer cell lines, rendering these cells resistant to drug-induced apoptosis.¹⁹

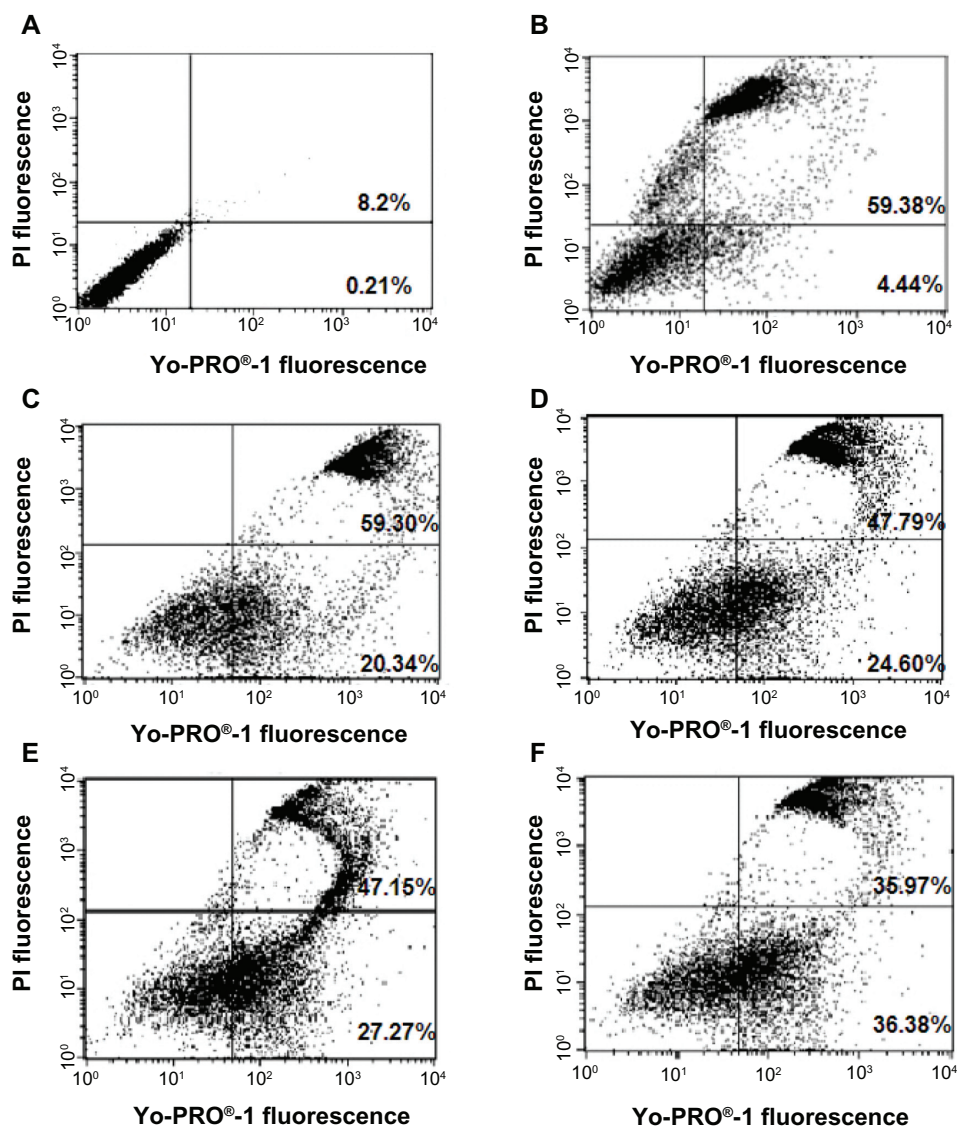


Figure 6 Flow cytometric dot plots showing status of wortmannin-induced apoptosis in MCF-7 cells. Yo-PRO®-1 and propidium iodide (Life Technologies, Carlsbad, CA) were used to stain the cells for FACS analysis. (A) Control cells (without wortmannin), and cells incubated with wortmannin at concentrations of (B) 50 nM, (C) 200 nM, (D) 500 nM, (E) 1 μ M, and (F) 2 μ M for 24 hours.

Several researchers have reported that an abnormal or deregulated PI3k-Akt signaling pathway plays a critical role in breast malignancies associated with multiple chemotherapeutic resistance.^{18,20–22} Therefore, selectively inhibiting PI3k-Akt expression in breast cancer cells using a small molecule inhibitor, such as wortmannin, might have potential therapeutic value. Thus, our results suggest a novel biomolecular target pathway for apoptosis induction by wortmannin to improve therapeutics for MCF-7 breast cancer cells.

It was determined that wortmannin-treated MCF-7 cancer cells show significant morphological and biochemical apoptogenic characteristics, as indicated by inhibition of growth, reduction in cell viability, condensation of chromatin, DNA fragmentation, and increased percentages of

both early and late apoptotic populations and membrane blebbing, and thus they appear to be undergoing apoptotic cell death.^{2,10,28,35} This appearance is positively correlated with drug concentration and apoptosis induction despite the absence of normal caspase 3 in the cells.^{23,24} It has been reported that wortmannin toxicity is more potent in DNA damaged cells than in nondamaged cells.^{36,37} This was confirmed by the finding of an increased amount of chromatin condensation on fluorescence microscopy and early apoptotic phase on flow cytometry-based dot plot analysis.

Wortmannin-induced cell death was confirmed to be apoptotic by FACS dot plot analysis using Yo-PRO®-1 and propidium iodide double-staining. The significant percent-

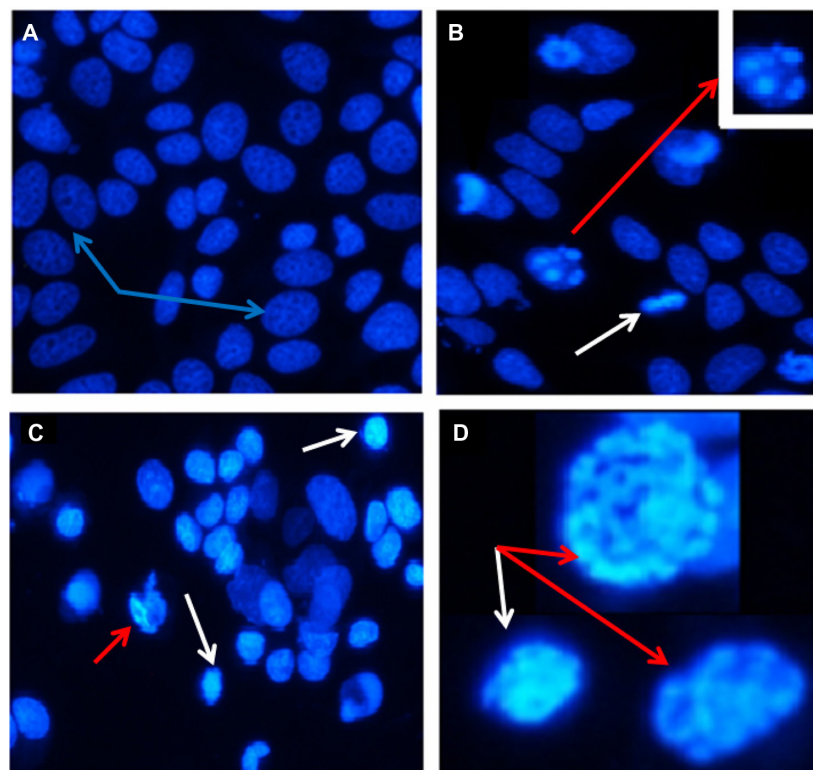


Figure 7 Wortmannin-treated MCF-7 cells demonstrating chromatin condensation effect. Representative nuclear staining of MCF-7 cells with Hoechst 33342. Changes in cellular nuclear morphology examined using fluorescence microscopy and a DAPI filter. **(A)** Control cells uniformly stained blue without condensed chromatin, with normal, round, and unpunctuated nucleus (blue arrow), recorded as nonapoptotic cells. **(B–D)** Cells with condensed and fragmented nuclei. Wortmannin-treated cells **(B)** show nuclear shrinkage, and chromatin and DNA condensation (white arrow), nuclear fragmentation with brighter blue-whitish (red arrow) fluorescent appearance compared with control, and were scored as apoptotic cells. **(C)** Percentage of chromatin condensation increases with higher concentrations of wortmannin. **(D)** Nuclear fragmentation in higher magnification.

age of cell death detected by flow cytometry is consistent with that detected by Trypan blue staining. In addition, morphological observation revealed typical apoptosis characteristics, including cell shrinkage, membrane blebbing, reduced cellular volume with condensed contents, and a

rounded body, further demonstrating wortmannin-induced apoptosis of MCF-7 cells. Moreover, qualitative MTT assay showed reduction of cell viability and inhibition of cellular proliferation by wortmannin in a dose-dependent manner. These effects are not significant at low concentrations, but

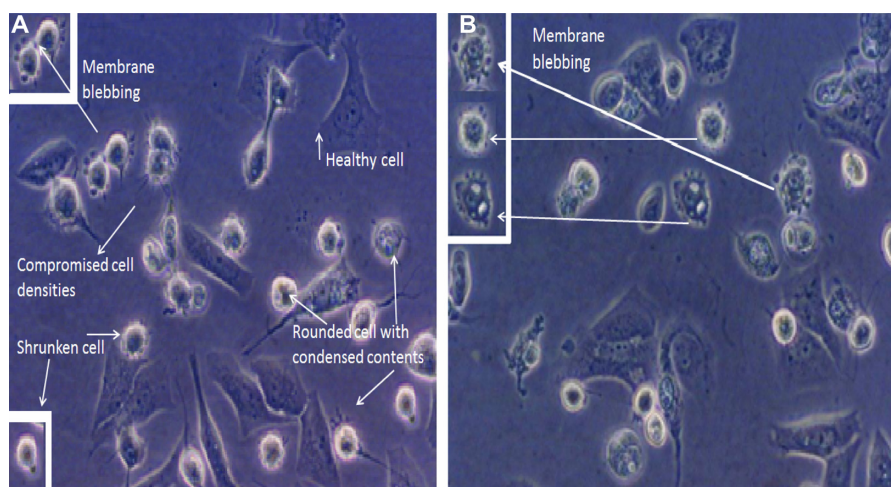


Figure 8 Phase-contrast micrographs of wortmannin-treated MCF-7 cells showing typical apoptotic morphological changes, eg, rounded, shrunken, and suspended cells, with apoptotic blebbing. **(A and B)** are representative of cells treated with wortmannin for 24 hours.

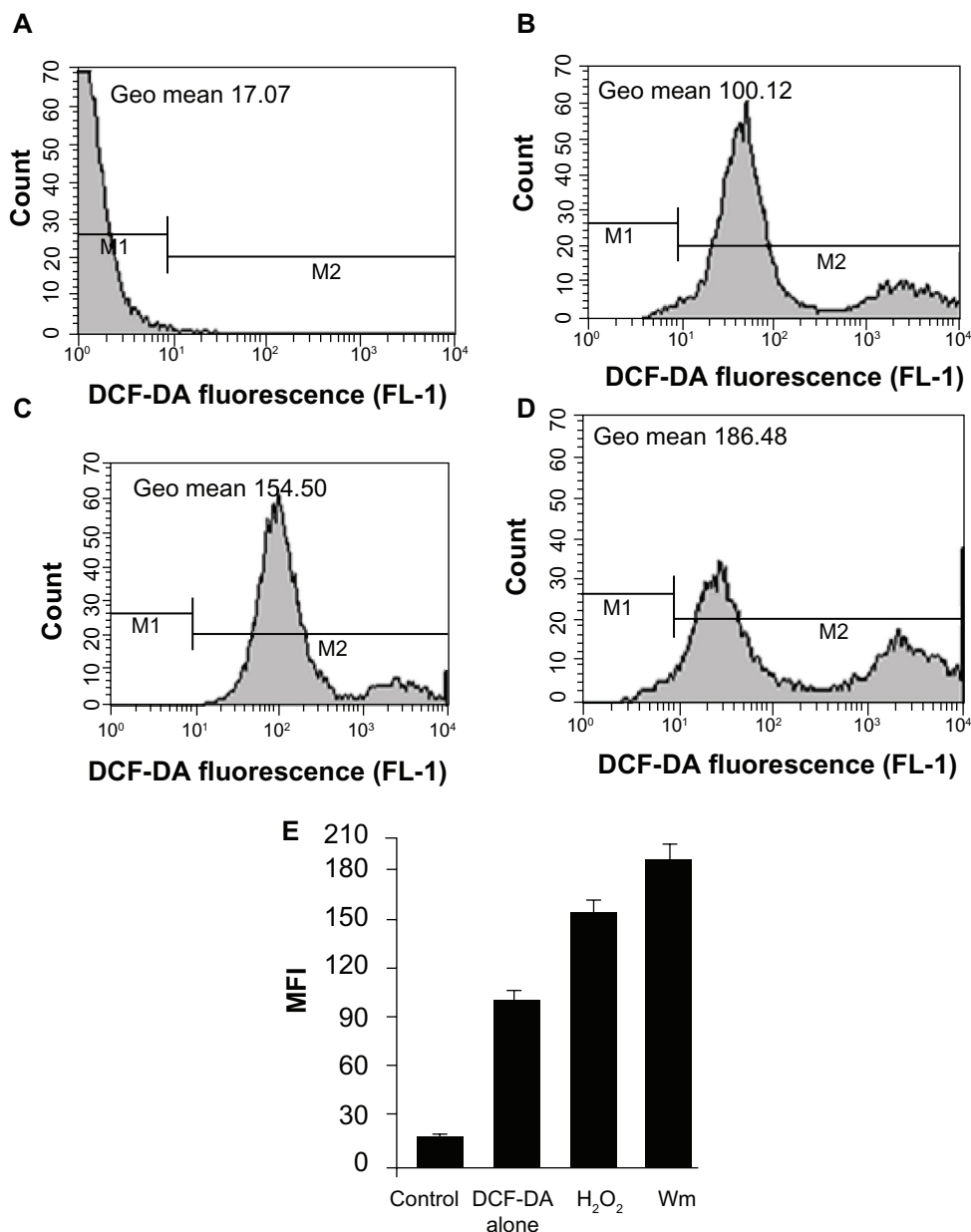


Figure 9 Intracellular accumulation of reactive oxygen species in MCF-7 cell lines. The x axis shows the fluorescent intensity of DCF-DA oxidation, and the y axis indicates cell numbers. (D) Histograms showing representative results of wortmannin-treated cells compared with untreated control cells (A and B). (C) H₂O₂ positive control. (E) Bar graph showing the percentage of cells from both groups (treated and not treated with wortmannin) with mean fluorescence intensity.

the effect was more evident and a significant amount of apoptosis induction was observed at higher concentrations. Morphological differences between normal and apoptotic cells can be observed by phase-contrast microscopy, while Trypan blue exclusion is used to evaluate cell death rates. Rapid and sensitive MTT assay qualitatively evaluates mitochondrial activity to test cell growth as well as cell death.³⁸

Several studies have reported that wortmannin has an antitumor effect in human breast cancer cell lines, and are inconsequential in the MCF-7 context, specifically from the

programmed cell death perspective.^{39–43} To the best of our knowledge, there are no further reports on the details of the mechanism of action on MCF-7 cell death. Therefore, we have explored for the first time the possible mechanism of cell death induced by wortmannin in MCF-7 cells by focusing on its ability to induce programmed cell death.

Chromatin condensation is one of the prominent hallmarks of apoptosis, and is brought about by activated caspases. To study whether the cytotoxicity of wortmannin in MCF-7 cells was associated with induction of apoptosis, we used the Hoechst 33342 chromatin condensation staining

method after treating cells with wortmannin for 24 hours. Wortmannin-treated cells (Figure 7B–D) showed nuclear shrinkage, condensation of chromatin and DNA, and nuclear fragmentation, with a brighter blue-whitish fluorescent appearance compared with controls. The changes in typical apoptotic nuclear morphology, such as DNA fragmentation, nuclear chromatin hypercondensation, and nuclear shrinkage, were identified in treated cells but not in untreated control cells. Normal nuclei contained noncondensed chromatin uniformly dispersed over the entire nucleus, and these cells were recorded as nonapoptotic cells, whereas cells containing condensed chromatin were scored as apoptotic cells.

ROS has been reported to have a dual mechanistic role in living cells. At low concentrations, ROS serve as important signaling molecules and at higher concentrations they induce cell death.^{2,29,44–49} Several studies have reported that increased ROS generation can selectively kill cancer cells by a more rapid increase in the toxic threshold compared with normal cells.^{44,45,50,51} In light of this, we investigated the possible role of ROS in wortmannin-treated MCF-7 cell lines using FACSCaliber. Our results show that wortmannin induced significant accumulation of ROS in cells. Thus, our results confirm the essential role of ROS in induction of apoptosis in MCF-7 cells.

Our morphological and biochemical analysis indicates that increased intracellular ROS production also subsequently promotes apoptotic cell death.^{49,51,52} These data are consistent with observations made by earlier researchers,^{11,17,34,53–55} and establish the anticancer properties of wortmannin in MCF-7 breast cancer cells. These findings could be used to translate the anticancer effects of wortmannin into other cancer cell lines too. Thus, our study could be useful for developing anticancer therapeutics based on programmed cell death in cancer cells resistant to apoptosis induced by other common chemotherapeutic drugs.

Overall, the present study shows the cytotoxic effects of wortmannin, in particular its effects on cell growth and induced apoptotic cell death in the MCF-7 breast cancer cell line. To the best of our knowledge, this is the first report of wortmannin-induced breast cancer cell toxicity and programmed cell death via the apoptosis pathway in a MCF-7 cancer cell line in vitro. Wortmannin has apoptotic properties that suggest potential use specifically in the MCF-7 cancer cell line. However, further investigation would be necessary to elucidate in detail the molecular signaling mechanisms involved in these apoptotic cell death pathways in MCF-7 and other cancer cell lines.

Disclosure

The authors report no conflicts of interest in this work.

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