Wortmannin inhibits proliferation and induces apoptosis of MCF-7 breast cancer cells

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Summary

Objective: The present study aimed to explore the effects of wortmannin in the proliferation and apoptosis of human breast cancer MCF-7 cells. *Methods*: The authors treated cells with 0, 1, 6.25, 12.5, 25, and 50 nM wortmannin for 24, 48, and 72 hours. Inhibition of proliferation was measured by cell counting kit-8 assay (CCK8). Apoptosis was detected with Annexin V-fluorescein isothiocyanate/propidium iodide double staining by flow cytometry. Additionally, expression of proteins involved in the PI3K pathway, specifically total Akt, phosphorylated Akt (p-Akt), and NF-κB was detected by Western blotting following 24 hours of wortmannin exposure. *Results:* Higher doses (6.25, 12.5, 25, and 50 nM) of wortmannin significantly inhibited proliferation of MCF-7 cells after 24, 48, and 72 hours of exposure compared with control MCF-7 cells incubated with DMSO alone in DMEM ($p < 0.05$). This inhibition increased with concentration and duration of treatment. Similarly, wortmannin at 6.25, 12.5, 25, and 50 nM concentrations significantly increased apoptosis of MCF-7 cells following 24 hours of exposure ($p < 0.05$). Western blotting revealed that increasing concentrations of wortmannin (6.25, 12.5, 25, and 50 nM, 24 hours) increasingly reduced expression of p-Akt and NF-κB; however, expression of total Akt was unaffected at any concentration of wortmannin. *Conclusions:* Wortmannin inhibits proliferation and induces apoptosis of MCF-7 cells in a dose and time-dependent manner, likely through down-regulation of PI3K/Akt signaling and NF-κB protein expression.

Key words: Wortmannin; MCF-7 cell line; Proliferation; Apoptosis; PI3K/Akt.

Introduction

The phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway is critical for cell proliferation and differentiation and commonly exhibits changes in tumor cells [1-3]. Indeed, this pathway plays important roles in maintaining the biological characteristics of malignant cells. Further, PI3K/Akt signaling may aid tumor cells in escaping damage produced by anti-cancer drugs [1-3]. Thus, inhibitors of this pathway have been heavily investigated for their potential to block tumorigenesis.

One such PI3K inhibitor is wortmannin. Isolated from *penicillium wortmannii* in 1957, wortmannin is a steroid metabolite belonging to the viridin family. This compound specifically inhibits catalytic activity of PI3K through the p110 subunit, blocking activation of the PI3K/Akt pathway and thereby inhibiting cell proliferation [4]. Many studies have shown that wortmannin has broad-spectrum anti-fungal activity and anti-inflammatory activities [5], and at least one study has demonstrated its anti-tumor activity [6]. Recent studies have employed wortmannin to treat various advanced tumors, including lung [7] and gastric cancer [8, 9], revealing its potential for cancer therapy.

The PI3K/Akt pathway is crucial in the development and progression of breast cancer, and PI3K inhibitors have been heavily investigated in this setting [10]. However, wortmannin has not been extensively studied for its ability to prevent or treat breast tumors. Therefore, a study was designed to investigate the effects of wortmannin in the proliferation and apoptosis of MCF-7 breast cancer cells positive for estrogen receptor, to determine a potential clinical application for this disease.

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Materials and Methods

Cell culture

The human breast cancer MCF-7 cell line was purchased from the Cell Bank of Typical Model Cultivation Preservation Committee, Chinese Academy of Sciences. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 0.01 mg/ml bovine insulin at 37ºC with 5% CO_2 for ten generations.

Wortmannin administration in MCF-7 cells

MCF-7 cells in the logarithmic growth phase were harvested to 1×10^4 cells/ml in single-cell suspension, with 100 µl inoculated into each 96-well culture plate. The medium was extracted and discarded 24 hours later. For each experimental group, six parallel wells were used. To each well 100 µl wortmannin (Alexis Corp., Switzerland) solution, dissolved in DMSO (Sigma) at concentrations of 1, 6.25, 12.5, 25, and 50 nM, were added. Control wells received 0.1% DMSO in DMEM. Cells were then cultured for 24, 48, or 72 h.

Detection of cell proliferation

The colorimetric cell counting kit (CCK8; Dojindo, Japan) was used according to manufacturer's instructions to determine cell proliferation and viability following wortmannin administration. Absorbance, (A), was measured on a microplate reader at 450 nm optical spectrum. Values were determined three times for each sample, and the average value was used to calculate the inhibition rate. Proliferation inhibition rate = [1 - (experimental group A value/control group A value)] × 100%. The experiment was performed in triplicate.

Detection of apoptosis

Twenty-four hours after wortmannin or DMSO/DMEM administration, EDTA-free trypsin (Life Technologies, Inc.) was used to detach cells for harvesting. Cell suspensions were centrifuged at 1000 rpm for five minutes and washed twice with PBS. Apoptosis was detected by addition of 5 µl AnnexinV-fluorescein isothiocyanate (FITC) and 5 µl propridium iodide (PI)

(both from BD Biosciences) for 15 minutes, followed by fluorescence-activated cells sorting (FACS) Canto flow cytometry (BD Biosciences). The experiment was repeated three times. Early apoptosis rate was defined as the percentage of FITC+/PI- cells, and the late apoptosis rate defined as the percentage of FITC+/PI+ cells.

Western blotting

Twenty-four hours after wortmannin or DMSO/DMEM administration, MCF-7 cells were harvested to dilutions of 1×10^7 cells. Cells were pre-cooled at 4ºC and washed twice with PBS. 100 µl cell lysis solution were incubated with suspensions in a water bath for 30 minutes, then suspensions were centrifuged at 12000 × g at 4ºC for eight minutes. Supernatants were collected and proteins were quantified via Bradford method. Fifty µg of protein was removed was added 2 x SDS loading buffer for protein denaturation for five minutes in boiling water. Fifty µg total protein was separated by SDS-PAGE (BIO-RAD) and transferred to nitrocellulose at 4ºC and 110 V. Membranes were treated with 5% nonfat milk at room temperature for 60 minutes. Primary antibodies (Cell Signaling Technology) against total Akt (rabbit anti-mouse monoclonal), p-Akt (rabbit anti-mouse monoclonal), or NF-κB p65 (human anti-mouse) were diluted 1 : 2000 and incubated with membranes overnight at 4ºC. Membranes were washed with 0.05% Tween-20 TBS. Secondary antibodies (goat anti-rabbit, labeled with horseradish peroxidase), as well as the internal control for GAPDH (rabbit anti-human; 10 ml diluted at 1 : 5000), were incubated with membranes at room temperature for 60 minutes. Staining was developed with DAB, images were captured with a digital camera, and analyzed with the GDS 8000 image analysis system. The ratio of mean optical density values of corresponding protein bands and GAPDH protein was used to express protein levels for each group.

Statistical methods

SPSS13.0 software was used for statistical analysis. Measurement data are expressed as mean ± standard deviation ($\bar{x} \pm s$). Single factor ANOVA was used to compare inhibition rates, apoptosis rates, and protein expression levels using different concentrations of wortmannin on MCF-7 cells and to perform pairwise comparison between groups (SNK method). The above analysis was performed with two-sided tests, with level of 0.05, and $p < 0.05$ considered statistically significant.

Results

Wortmannin inhibited MCF-7 cell proliferation

Addition of wortmannin to MCF-7 cell cultures at a concentration of one nM did not affect cell proliferation (Table 1), as detected by colorimetry. However, the addition of 6.25, 12.5, 25, or 50 nM wortmannin inhibited MCF-7 cell proliferation after 24, 48, and 72 hours ($p < 0.05$). Inhibition rates increased with increasing concentrations or time. Inhibition rates were significantly different ($p < 0.05$) from proliferation in the control group, which was exposed only to DMSO in DMEM. Pairwise comparisons between groups revealed the statistically significant differences with increasing doses ($p < 0.05$).

Wortmannin induced MCF-7 cell apoptosis

With annexin and PI to label cells undergoing programmed cell death, flow cytometry was used to assess levels of apoptosis of MCF-7 cells 24 hours following treatment with wortmannin. The mean percentage of apoptotic cells was not affected in cells treated with 1 nM wortmannin (Table 2). However, the percentages of early apoptotic MCF-7 cells (defined as annexin-positive/PI-negative) and late apoptotic MCF-7 cells (annexin-positive/PI-positive) were significantly different for cells treated with 6.25, 12.5, 25, and 50 nM doses of wortmannin compared to their respective controls (all $p < 0.05$). Indeed, apoptosis increased with increasing concentrations of wortmannin. Additionally, percentages of cells undergoing early apoptosis were higher than those undergoing late apoptosis for 6.25, 12.5, 25, and 50 nM wortmannin.

Table 1. — *Effects of wortmannin in the proliferation of MCF-7 cells ($\bar{x} \pm s$).*

Wortmannin (nM)	Inhibition rate (%) at time point following treatment 24h	48h	72h
Control (DMSO only)	1.34 ± 0.09	1.44 ± 0.11	1.46 ± 0.09
1	2.33 ± 0.27	2.62 ± 0.25	2.51 ± 0.13
6.25	16.22 ± 1.13*#	32.51 ± 0.78*#	43.28 ± 2.72*#
12.5	26.48 ± 1.87*#□	41.42 ± 1.16*#□	44.89 ± 1.31*#
25	45.54 ± 1.47*#□▲	51.29 ± 0.76*#□▲	56.33 ± 1.24*#□▲
50	51.58 ± 1.49*#□▲○	55.73 ± 3.15*#□▲○	62.60 ± 2.27*#□▲○
F	890.593	808.936	809.716
p	0.001	0.001	0.001

*$p < 0.05$, *vs* Control group; #$p < 0.05$ *vs* 1 nM; □$p < 0.05$ *vs* 6.25 nM; ▲$p < 0.05$ *vs* 12.5 nM; ○$p < 0.05$ *vs* 50 nM.

Table 2. — *Apoptosis of MCF-7 cells after 24-hour culture with wortmannin ($\bar{x} \pm s$).*

Wortmannin (nM)	Rate of apoptosis (%) Early	Late	*t*	*p*
Control	1.40 ± 0.07	1.44 ± 0.07	0.705	0.520
1	1.75 ± 0.10	1.69 ± 0.15	0.549	0.612
6.25	14.82 ± 0.97*#	10.59 ± 0.86*#	5.655	0.005
12.5	16.38 ± 1.09*#	12.68 ± 0.67*#□	5.002	0.007
25	21.28 ± 0.99*#□▲	17.10 ± 0.37*#□▲	6.858	0.002
50	25.30 ± 1.35*#□▲○	21.13 ± 0.42*#□▲○	5.103	0.007
F	359.086	753.658		
p	0.001	0.001		

*$p < 0.05$, *vs* Control group; #$p < 0.05$ *vs* 1 nM; □$p < 0.05$ *vs* 6.25 nM; ▲$p < 0.05$ *vs* 12.5 nM; ○$p < 0.05$ *vs* 50 nM.

Wortmannin affected expression of proteins in the PI3K/Akt pathway

To assess the possibility that wortmannin may alter cell proliferation and induces apoptosis of MCF-7 cells via inhibition of the PI3K/Akt pathway, Western blotting was employed in cells treated with wortmannin using antibodies against three proteins that may be affected: Akt, phosphorylated Akt (p-Akt), and NF-κB p65, which appears to be activated by the PI3K pathway, particularly in cancer biology [11]. Expression of total Akt was not affected 24 hours after any exposure to wortmannin compared with control medium (Figure 1); however, increasing concentrations of wortmannin (6.25, 12.5, 25, and 50 nM) caused larger reductions in expression of both p-Akt and NF-κB p65.

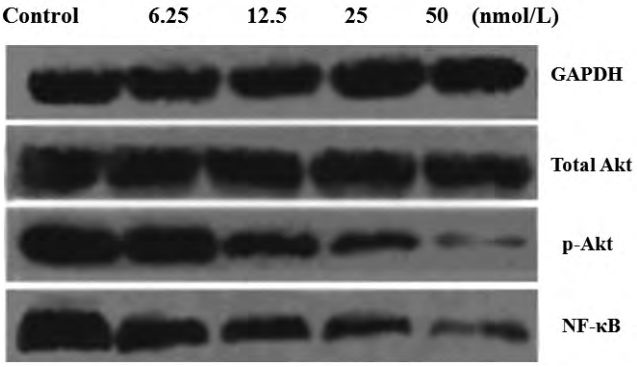

Figure 1. — Western blot for protein expression in MCF-7 cells after 24 hour culture with wortmannin at 6.25, 12.5, 25, and 50 nM doses. GAPDH served as an internal loading control. Fifty μg of total protein lysate was loaded in each lane. Antibodies against total Akt, phosphorylated Akt (p-Akt), and NF-κB p65 were hybridized and staining was quantified using mean optical density of bands.

Discussion

While the pathogenesis of breast cancer is complex and multifactorial, involving multiple genes and environmental interactions, cell signaling plays a vital role in its occurrence and development. The present study demonstrates that breast cancer cells show reduced proliferation after addition of wortmannin, an inhibitor of the PI3K/Akt signaling pathway. These effects in cell proliferation were increasingly evident at higher drug concentrations. Concurrently, wortmannin exposure induced apoptosis of MCF-7 cells. Apoptosis significantly increased with increasing concentration of the drug, consistent with other reports [12].

Because wortmannin is known to specifically inhibit the PI3K/Akt pathway, the authors investigated whether alterations in this pathway might be responsible for the reduced proliferation and increased apoptosis of MCF-7 cells following wortmannin exposure. p-Akt expression decreased with increasing concentrations of wortmannin; however, total Akt expression was not affected at any concentration. This finding indicates that only the active (phosphorylated) form of Akt was affected by wortmannin activity. Therefore, the authors hypothesize that the anti-proliferative activity of wortmannin is correlated with PI3K/Akt signaling pathway activation.

The transcription factor NF-κB is considered a key molecule in regulating oncogene expression, enabling apoptosis inhibition of tumor cells while also promoting metastasis and drug resistance [13]. This protein however, is also an important link in signal transduction pathways, making it an ideal target for cancer prevention and enhancing sensitivity to chemotherapy [14]. This protein is also believed to interact with PI3K/Akt [11]. In this instance, wortmannin exposure down-regulates NF-κB protein levels in MCF-7 cells, demonstrating that inhibition of PI3K by this drug also affects the NF-κB signaling pathway. This finding is supported by recent literature [15, 16]. Poh *et al*. [16] studied the effects of wortmannin on the PI3K/Akt pathway, demonstrating that the drug negatively regulates multiple molecules in the pathway, including PI3K and mTOR, to dephosphorylate Akt and reduce its activity. The present findings indicate that further investigation of the link between NF-κB and Akt signaling is required.

In summary, wortmannin exposure significantly inhibits proliferation and induces apoptosis of MCF-7 breast cancer cells. Such changes may inhibit tumor cell growth and differentiation and reduce the invasiveness of tumor cells via inhibition of the PI3K/Akt signal transduction pathway. These findings suggest that wortmannin offers potential in anti-tumor targeted therapy and drug screening for breast cancer.

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