

Wortmannin enhances cisplatin-induced apoptosis in human ovarian cancer cells *in vitro*

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Abstract. – OBJECTIVE: The PI3K/Akt signaling pathway is constitutively activated in some ovarian cancers; when activated, it promotes invasion and inhibits chemotherapy-mediated apoptosis in cancer cells. The fungal metabolite wortmannin is the currently known inhibitors that show fairly high specificity for PI3K. We examined whether PI3K/Akt activity correlates with invasion and apoptotic resistance to chemotherapy in cultured human ovarian cancer cells, and whether inhibition of PI3K/Akt by wortmannin inhibits invasion and enhances cisplatin-induced apoptosis in cultured human ovarian cancer cells.

MATERIALS AND METHODS: The cisplatin-sensitive A2780 ovarian adenocarcinoma cell line and its daughter line, A2780cis was evaluated for basal Akt activity. Chemotherapy-induced cell death was evaluated following down-regulation of Akt activity by wortmannin treatment or upregulation of Akt activity by myr-Akt treatment. Invasion and migration were assessed using Boyden chamber assays

RESULTS: Inhibiting or activation of PI3K/Akt signaling pathway by wortmannin had little effect on the basal level of apoptosis in ovarian cancer cells, but increased the apoptotic effect of chemotherapy in A2780cis cells, decreased the apoptotic effect of chemotherapy in cisplatin-sensitive A2780 cells. Cisplatin resistant cells display increased potential for migration and invasion.

CONCLUSIONS: The antiapoptotic effect of AKT activation in ovarian cancer cells confer invasive ability and resistance to apoptosis. Wortmannin is as adjuncts to conventional chemotherapy in the treatment of ovarian cancer.

Key Words:

Homocysteine (Hcy), Atherosclerosis (AS), Oxidative stress, Lutein.

Introduction

Ovarian cancer has the highest associated mortality rate in the western world^{1,2}. While relatively 1/70 women affected in China³, approxi-

mately 70-80% of patients with ovarian cancer will succumb to the disease within five years of diagnosis⁴. The high mortality rate is due, in part, to the fact that ovarian cancer is often diagnosed in advanced stage, because of a lack of measurable early symptoms and ineffective screening techniques^{5,6}. Of equal importance, 20% of tumours display primary resistance to platinum compounds while the majority of initial responders will relapse, often as a result of acquired drug resistance^{7,8}.

Standard treatment for ovarian cancer involves tumour debulking and platinum-based chemotherapy administered intravenously or intraperitoneally^{9,10}. Cisplatin, the most common first line chemotherapeutic drug, is a platinum compound that binds to and crosslinks DNA¹¹. During cell division cisplatin-DNA adducts block replicative machinery, inducing the DNA damage response, and eventually apoptosis^{11,12}. It has been proposed that decreased cellular uptake of drug as well as increased capacity for DNA damage repair and anti-apoptotic signaling may play a role in cisplatin resistance displayed by many tumours¹³⁻¹⁷.

The phosphatidylinositol-3 kinase (PI3K)/Akt is a fundamental signaling pathway that mediates several cellular processes, including cell proliferation, growth, survival, and motility¹⁸⁻²⁰. Increased activation, deregulation, and mutation of the components in the PI3K/Akt pathway have been implicated in driving tumorigenesis and conferring resistance to chemotherapy²¹⁻²³. Akt (protein kinase B) is a well characterized serine/threonine kinase that is the central protein in the PI3K/Akt signaling pathway. Increased Akt activity has been demonstrated in many types of cancer, where it transmits a potent survival/anti-apoptotic signal²⁴.

Elevated levels of phosphorylated Akt can protect cells from undergoing apoptosis induced by

cytotoxic drugs and contribute to drug resistance. Given that the most prevalent genetic aberrations found in ovarian cancer are capable of up-regulating the activity of the PI3K-PKB/Akt cell survival pathway, we hypothesize that its perturbation by pharmacological inhibitors may have therapeutic potential.

The fungal metabolite wortmannin is the currently known inhibitors that show fairly high specificity for PI3K. Wortmannin binds to the p110 catalytic subunit of PI3K, noncompetitively and irreversibly inhibiting (IC₅₀, 2-4 nM) the enzyme²⁵. It has been shown that wortmannin at 100 nM causes 95% inhibition of PI3K and that the concentration required to induce apoptosis of cells maintained in growth factors correlates closely with that required for PI3K inhibition²⁶. Wang et al²⁷ has found, the activation of Akt was required for cold exposure-induced ATP elevation. Blockade of Akt activation by wortmannin diminished the transient increase of intracellular ATP content and exacerbated cell apoptosis during acute cold exposure. Yu et al²⁸ has found, wortmannin inhibits proliferation and induces apoptosis of MCF-7 cells in a dose and time-dependent manner. Kim et al²⁹ has found that in murine hepatocarcinoma, the anti-tumor effect of radiation was potentiated by wortmannin. The mechanism seems to involve not only the increase of induced apoptosis but also enhanced vascular injury. Wortmannin, in combination with radiation therapy, may have potential benefits in cancer treatment. However, it is unclear whether the wortmannin could suppress growth, invasion and enhances cisplatin-induced apoptosis in ovarian cancer.

The current study examined the *in vitro* effects of wortmannin on Akt phosphorylation, invasion and apoptotic effect of chemotherapy. Wortmannin invasion and sensitises ovarian cancer cells to the apoptotic effect of cisplatin.

Materials and Methods

Cell Lines and Culture Conditions

The cisplatin-sensitive A2780 ovarian adenocarcinoma cell line and its daughter line, A2780cis, were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in Roswell Park Memorial Institute (RPMI) with 10% FBS (Fetal Bovine Serum), 1% penicillin/streptomycin and 1% L-glutamine at 37°C in 5% CO₂. A2780cis cells were main-

tained in media with 1 µM cisplatin. For all assays, cells were grown to 70-80% confluence and harvested following trypsinization.

Drug Treatments

For flow cytometric analysis, cells were treated with the drug vehicle (1% dimethyl sulfoxide, DMSO) or 10 µM cisplatin for 48 h or with the same concentration of cisplatin for the same duration followed by wortmannin (00 µM) for 4 h in the continuous presence of cisplatin. The concentration and duration of cisplatin treatment were chosen based on preliminary studies examining its effects on cell cycle inhibition and induction of apoptosis. For Western blotting, cells were treated with similar concentrations of wortmannin alone for 4 h or with 10 µM cisplatin alone for 48 h before harvest. Wortmannin was purchased from Biomol (Philadelphia, PA, USA). All compounds were dissolved in DMSO at a stock concentration of 10 mM, stored at -20°C, and added to cell cultures at a final concentration of #1% DMSO, with appropriate solvent additions to control cultures. All experiments were performed in triplicate.

Modulation of Akt Activity

In order to demonstrate the role of Akt in response to chemotherapy, modulation of basal Akt activity was performed. Akt expression was augmented through the transient transfection of constitutively active myristylated Akt1 (myr-Akt) cDNA in pUSEamp (Upstate, Charlottesville, VA, USA). In brief, A2780 cells were plated to a density of 5 × 10⁴ cells/ml. After allowing 24 hours for cellular recovery and adherence, cells were transfected with 1 µg of the myr-Akt plasmid in association with 5 µl of Lipofectin reagent in serum-deprived media. Approximately 12-16 hours following transfection, serum containing media (DMEM+10% FBS) was reintroduced and cells were incubated for an additional 48 hours. Increases in Akt expression were demonstrated via Western blotting as previously described. Treatment with 10 µM cisplatin was initiated at this 48 hour time point when Akt activity was known to be at its peak.

Western Blotting

Following various treatments, cells were harvested by trypsinisation (trypsin 0.25% w v⁻¹, 1 mM ethylenediaminetetraacetic acid). The cells were lysed in a lysis buffer containing 150 mM NaCl, 1% Triton X-100 and 25 mM Tris (pH

7.5). Debris was sedimented by centrifugation for 5 min at 12 000 g, and the supernatants were solubilised for 5 min at 100°C in Laemmli's sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 100 mM dithiothreitol. Protein concentrations of the lysates were determined with a protein quantitation kit (Bio-Rad Laboratories, Hercules, CA, USA), and 40 mg of each sample was separated on a 10% SDS-PAGE gel. Separated polypeptides were then electrophoretically transferred to 0.2-mm nitrocellulose membranes. Membranes were blocked for 1 h in a Tris-buffered saline-Tween (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20) containing 5% (w v⁻¹) nonfat dried milk. The blots were then probed overnight with primary antibodies and developed using species-specific secondary and tertiary antisera. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amersham, Harlington Heights, IL, USA).

Boyden Chamber Migration and Invasion Assays

Cells were serum starved for 24 hours prior to use. Media with 10% FBS was added to the wells of a 24-well plate. BD Falcon™ Cell Culture Inserts (BD Biosciences, Mississauga, Canada) were placed in each well. 2×10^3 cells in serum-free media were added to the interior of each insert. Plates were incubated for 24 hours at 37°C in 5% CO₂, and media removed from the insert, which was then washed with PBS (phosphate buffered saline). Insert membranes were fixed with cold methanol for 10 minutes, stained with 0.5% Crystal Violet in 25% methanol for 10 mins and rinsed with water to remove excess dye. Membranes were removed from the insert, placed under a microscope and the number of cells that migrated through the porous membrane was counted. Invasion assays were done as described above using BD BioCoat™ Matrigel™ invasion chambers (BD Biosciences, Mississauga, ON, Canada).

FACS Analysis

To identify and quantitate changes in the cell cycle distribution and the induction of apoptosis, treated cells underwent propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS). In brief, cells were plated at a density of 1×10^5 cells/ml. After allowing 24 hours for cell adherence, cells were transfected and/or treated. Cells were collected by gentle trypsinization,

washed in phosphate-buffered saline (PBS), pelleted by centrifugation and fixed in 70% ethanol. Immediately prior to staining, cells were washed twice in PBS and resuspended in PBS containing RNase A (20 µg/ml). Cells were stained with propidium iodide (final concentration 10 µg/ml) for 10 min at room temperature. Samples were analyzed by FACS (FL-3 channel) using a Beckman Coulter Counter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). For each sample, 50,000 events were collected and stored for subsequent analysis using EXPO software (version 2.0; Applied Cytometry Systems, Sheffield, UK). The percentage of cells in the sub-G₀ phase was quantitated as an estimate of cells undergoing apoptosis.

Statistical Analysis

All values are presented as mean ± SE. Statistical significance of all data was evaluated using the Student's *t*-test, $p < 0.05$.

Results

Basal Expression of AKT Differs Among A2780 and A2780^{cis} Ovarian Adenocarcinoma Cell Line

Constitutive activation of AKT has been reported in many cancers, including breast, ovarian, and prostate²⁴. The first step in this investigation was to analyze the basal expression of AKT in A2780 and A2780^{cis} ovarian adenocarcinoma cell line. Cell lysates from A2780 and A2780^{cis} ovarian adenocarcinoma cell line were screened by western blotting for the level of pSer⁴⁷³ AKT and total AKT. A2780^{cis} demonstrated high levels of AKT activation as determined by pSer⁴⁷³ AKT levels, while A2780 showed low levels of AKT activation (Figure 1). Total Akt level was variable and did not necessarily correlate with the levels of pSer⁴⁷³ Akt.

Effect of Cisplatin Exposure on Activation of Akt

As shown in Figure 1, cisplatin treatment was observed to induce a rapid increase in pSer⁴⁷³ Akt levels in A2780 cells over a time frame of 15 minutes to 6 hours. No significant activation of Akt in A2780^{cis} ovarian adenocarcinoma cell line was observed following any of the treatments (Figure 1). Total Akt level did not change compared to the control.

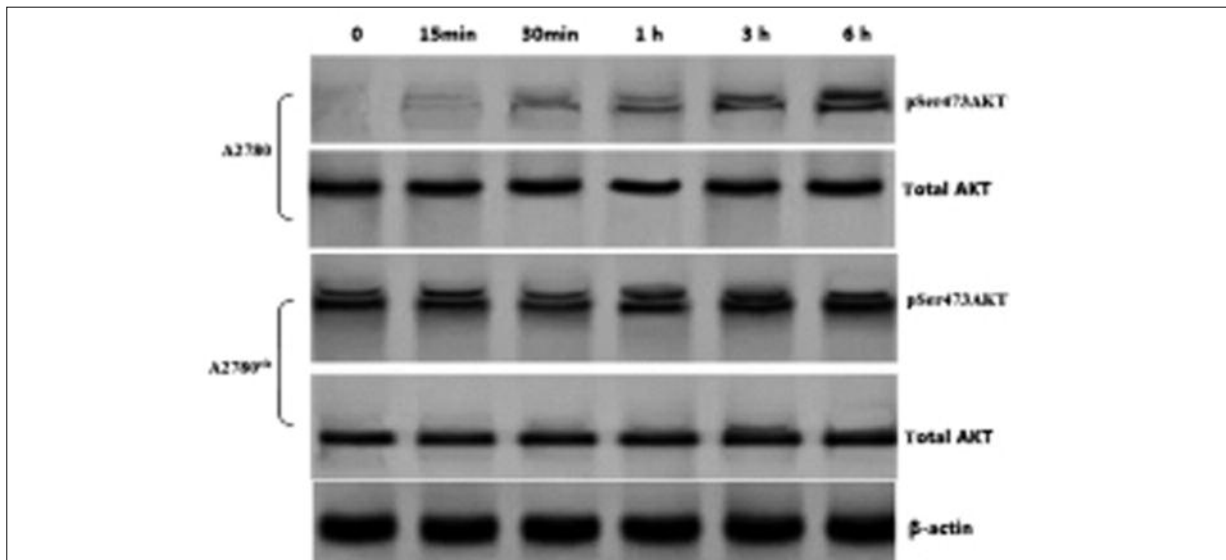


Figure 1. Western blot following the indicated times of treatment following cisplatin (10 μ M) in A2780 and A2780^{cis} ovarian adenocarcinoma cell line for phospho-Akt (pSer⁴⁷³) or β -actin to show for equivalency of loading.

Increasing AKT Activity by myr-Akt Decreases Cisplatin-induced Apoptosis in A2780 Cell

It was sought to increase Akt expression in the A2780 cells (low Akt activation) to determine if the cisplatin response would be more like that of A2780^{cis} (high Akt activation). The A2780 cells were transfected with a plasmid that encodes a constitutively active Akt (myr-Akt) and cells were analyzed by Western blot for pAkt levels (Figure 2 A). Increasing Akt activity in A2780

had no effect on basal levels of cell death (Figure 2B). When Akt activity was increased by the myr-Akt transfection, cisplatin treatment led to decreased cell death. Following cisplatin treatment, apoptotic fraction decreased from 48.6% to 14.7% ($p < 0.05$; Figure 2 B). However, increasing Akt activity did not alter the cellular response to cisplatin in A2780^{cis} cells (data not shown). Therefore, while increasing Akt activity in A2780 made the cellular response of A2780 more similar to the high-Akt A2780^{cis} cell line.

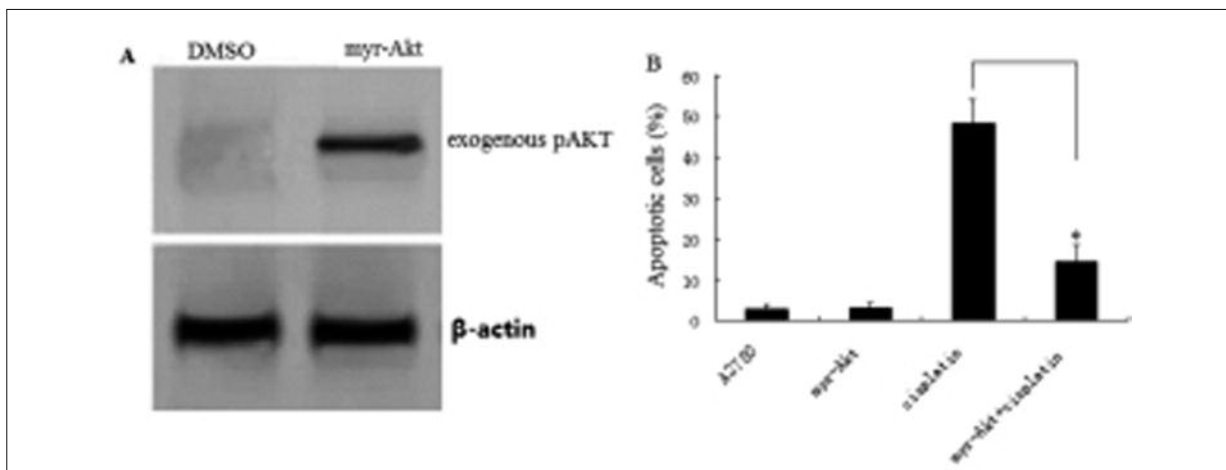


Figure 2. The effect of AKT activity by myr-Akt on cisplatin-induced apoptosis. **A**, Western blot illustrating a increase in phospho-Akt levels following transfection of 1 μ g myr-Akt in A2780 cells. **B**, Apoptotic fraction of A2780 cell line following treatment with cisplatin (10 μ M, 48 hours) in the absence or presence of myr-Akt transfection ($p < 0.05$).

Decreasing AKT Activity by Wortmannin Increases Cisplatin-Induced Apoptosis

Wortmannin (400 nM) treatment inhibited Akt activity in A2780^{cis} cells (Figure 3 A); Furthermore, wortmannin (400 nM), when added to cisplatin-treated A2780^{cis} cells for 4 h, significantly increased the population of the cells (Figure 3 B). The wortmannin alone did not increase apoptosis in the A2780^{cis} cells.

Cisplatin Resistant Cells Display Increased Potential for Migration and Invasion

Migratory capacity was measured using Boyden chamber assays. In three independent experiments, 9.6 ± 1.8 drug sensitive A2780 cell and 52.4 ± 2.7 drug-resistant A2780^{cis} cell migrated through the membrane after 24 hours ($p = 0.007$). Resistant cells also showed a five fold increased invasive capability relative to sensitive cells using a Matrigel-coated Boyden chamber assay. After 24 hours, an average of 33.2 ± 4.4 sensitive cells and 192.7 ± 5.18 resistant cells invaded the matrix ($p = 0.007$).

Migratory capacity of the myr-Akt transfected A2780^{cis} cells was reduced from 52.4 ± 2.7 (untransfected controls) to 21.3 ± 2.8 cells migrating through the membrane ($p = 0.003$). Invasive capability was reduced from 192.7 ± 5.18 (untransfected controls) to 47.5 ± 5.2 cells invading the matrix ($p = 0.02$). Migratory capacity of the wortmannin (400 μ M) treated A2780 cells was increased from 9.6 ± 1.8 (untreated controls) to 34.2 ± 11.8 cells migrating through the membrane ($p = 0.001$). Invasive capability was increased from 33.2 ± 4.4 (untreated controls) to 123.3 ± 11.6 cells invading the matrix ($p = 0.02$).

Discussion

Ovarian cancer exhibits a high rate of platinum sensitivity in the first-line setting, but resistance frequently develops in recurrent disease³⁰. As such, understanding the signaling networks that regulate chemo resistance is critical for successful treatment. This can be said of any cancer commonly treated with cisplatin such as small cell lung cancer, head and neck cancer, colorectal and hepatocellular cancers.

Akt plays an important role in cancer therapy by promoting resistance to the apoptosis-inducing effects of chemotherapy^{31,32}. Thus, Akt-targeted molecular therapy has become an intense area of research in ovarian cancers³³⁻³⁵. Enthusiasm for this approach has been based on results obtained from pharmacologic inhibition of PI3K, the upstream activator of Akt³⁶. Only recently, were specific small molecule inhibitors of Akt developed in an attempt to avoid the toxicity of PI3K inhibitors (i.e. LY294002 or wortmannin). Yet the background identification of tumor or chemotherapy selection in ovarian cancer has not been reported to provide the essential background for further preclinical development of Akt inhibitors.

The results of the present study illustrate the variable expression and activity of Akt in the cisplatin-sensitive A2780 ovarian adenocarcinoma cell line and its daughter line, A2780^{cis}. High-Akt activity was found in the A2780^{cis} cell, and low-Akt activity was found in the A2780 cell. Furthermore, exogenously increasing Akt activity by myr-Akt transfection in A2780 cells confer resistance to cisplatin-induced cell death. Furthermore, inhibition of Akt activity by wortmannin

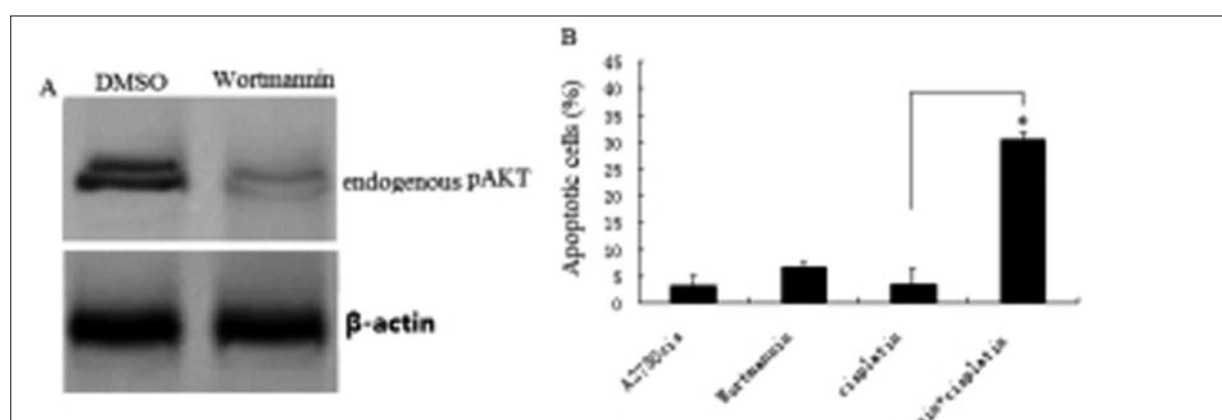


Figure 3. Wortmannin increases cisplatin-induced apoptosis. **A**, Western blot illustrating a decrease in phospho-Akt levels following wortmannin treatment in A2780^{cis} cells. **B**, Apoptotic fraction of A2780^{cis} cell line following treatment with cisplatin (10 μ M, 48 hours) in the absence or presence of wortmannin treatment ($p < 0.05$).

treatment increases cisplatin-induced apoptosis. The disparate results between the high-Akt and low-Akt cell lines related to cellular effects of cisplatin in combination with Akt inhibition warrant critical interpretation. These data would suggest that high levels of Akt activity may be a useful predictive marker for targeted Akt inhibition in combination with cisplatin.

The activated AKT kinase is necessary for many events of the metastatic pathway including escape of cells from the tumor's environment, into and then out of the circulation, activation of proliferation, blockage of apoptosis, and activation of angiogenesis. A series of steps leading to metastatic properties can be initiated upon activation of AKT by phosphorylation on Ser⁴⁷³. Our results demonstrate that the A2780^{cis} cell with high AKT activity, display increased potential for migration and invasion, and A2780 cell with low AKT activity, display decreased potential for migration and invasion. Inhibition of the AKT activity by wortmannin (400 μM) decreased the migration and invasion potential in the A2780^{cis} cell, and increasing Akt activity by myr-Akt transfection in A2780 cells increased the migration and invasion potential. Our results demonstrate for the first time that the prototype PI3K inhibitor wortmannin suppresses Akt phosphorylation and invasion potential.

Conclusions

We have demonstrated, through the use of an ovarian cancer cell line, A2780, and its cisplatin resistant daughter line, A2780^{cis}, that AKT activity directly contribute to cisplatin-resistance in ovarian cancer. We conclude that wortmannin inhibits Akt phosphorylation, invasion and enhances apoptosis induced by cisplatin in ovarian cancer cells *in vitro*. Our findings further support the pre-clinical development of treatment protocols that target the PI3K/Akt cell survival pathway to be used in ovarian cancer patients.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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