Introduction

As a serious and under-recognized tumor, ovarian cancer is the most lethal gynaecological malignancy and is the fourth most frequent cause of cancer-related deaths in women around the world. The high mortality is due to the fact that ovarian cancer often manifests with little or no specific symptoms at the early stage. The primary treatment for ovarian cancer is surgical resection of visible disease followed by adjuvant chemotherapy, which usually consists of a combination of platinum-based and taxane-based chemotherapy. More than 70% of such patients initially respond to cisplatin. However, the five-year survival rate is less than 25%. The high mortality rate is based on high proportion of advanced stage cases or aggressive cases experiencing chemoresistance and recurrence (1). For example, the 5-year survival rate of ovarian cancer cases at stage III and IV is about 28 and 16%, while the 5-year survival rate of stage I cases is up to 80% (2). Therefore, it is necessary to explore new approach to reverse chemotherapy resistance and to enhance sensitivity to platinum-based chemotherapy drugs.

The activation of PI3K/AKT/mTOR pathway can lead to an increase in cell proliferation, migration, invasion, and chemotherapy resistance of many cancers. For NSCLC patients, the aberrant activation of the PI3K/Akt/mTOR pathway is related to the resistance to gefitinib and a poor prognosis (3). For breast cancer, the activation of PI3K/AKT/mTOR pathway could increase the resistance of breast cancer cell clones to letrozole and develop an early resistance to letrozole in neo-adjuvant treatment for the patients (4). In ovarian cancer, the PI3K/AKT/mTOR pathway plays a key role in ovarian cancer tumorigenesis, progression, and chemotherapy resistance via the mutated or altered PI3K/AKT/mTOR pathway (5). In addition, the inhibition of the PI3K/Akt/mTOR pathway combined with classical anticancer agents is highly effective in several experimental studies (6), which could be the most appropriate approach for target therapy and to avoid acquired resistance in clinical.

This study aimed to evaluate the changes in cell cycle, apoptosis and the PI3K/AKT/mTOR pathway in ovarian carcinoma cells, SKOV3 and SKOV3/DDP, treated with PI-103 and cisplatin, and thus to explore the possible mechanism that PI-103 enhances the sensitivity of SKOV3/DDP ovarian cancer cell line to cisplatin.
Materials and methods

Cells and culture

Human ovarian cancer cells SKOV3 and the drug resistant human ovarian cancer cells SKOV3/DDP were obtained from Obstetrics and Gynecology Laboratory, Nanjing Medical University. RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) was used to culture cells. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. Cells from exponentially growing cultures were used in all experiments.

Chemicals and antibodies

PI-103 were purchased from Cayman company (United States) and cisplatin (DDP) was purchased from Shandong Qilu Pharmaceutical Factory, which were dissolved in RPMI 1640 medium in the concentration of 1 g/L. CCK-8 kit, cell cycle and apoptosis detection kit were purchased from Beyotime biotechnology company (Jiangsu). FBS was purchased from Hangzhou Sijiqing Company, and RPMI 1640 medium was purchased from Gibco Company. Flow cytometry (FACS Vantage SE) was purchased from BD Company. Anti cyclin D1, anti p21 and anti-cleaved caspase-3 were purchased from CST Company. Anti Akt and anti Phospho-Akt (Ser473) were from Bioworld Company. Anti rps6 and anti Phospho-rps6 were from CST Company. Anti β-actin was purchased from Sigma Company.

The inhibition rates of SKOV3 and SKOV3/DDP treated with different concentration of PI-103

The growth inhibition rates of SKOV3 and SKOV3/DDP cells were assessed using the CCK8 assay. Briefly, the exponentially growing cells were digested, and single cell suspension was obtained. A total of 10,000 cells were plated in 96-well flat bottom plates. After 24 hours incubation, the culture media was replaced by fresh culture media containing PI-103 in concentrations of 0.022, 0.044, 0.088, 0.175, 0.35, 0.70 and 1.4 mg/L. CCK8 (20 μL/well) solution was added into each well and incubated for 2 h at 37 °C. After incubation, absorbance was determined by a microplate reader at 450 nm and absorbance values were expressed as the percentage of the untreated control. The test was repeated in triplicate. The growth inhibition rate of SKOV3 and SKOV3/DDP was calculated as follows:

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\text{Inhibition rate} = \left(1 - \frac{\text{PI-103 group OD}}{\text{control group OD}}\right) \times 100\%.
\]

IC50 of DPP on SKOV3 and SKOV3/DDP

The IC50 of DPP for SKOV3 and SKOV3/DDP was assessed using the CCK8 assay. The cells were cultured in 96-well flat bottom plates as the same way described above, and the following concentration series of cisplatin was adopted, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00 and 100.00 mg/L. After 72 hours treatment, the inhibition rate was assessed using CCK8 assay, and IC50 was counted according to the concentration of cisplatin and the inhibition rate. The test was repeated in triplicate.

The effect of PI-103 on the IC50 of DPP for SKOV3 and SKOV3/DDP

The IC50s of cisplatin for SKOV3 and SKOV3/DDP treated with different concentrations of PI-103 were comparatively studied to evaluate the impact of PI-103 on the drug resistant. The cells were cultured in 96-well flat bottom plates as the same way described above, and then were treated with cisplatin and PI-103. The concentration series of cisplatin adopted were 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00 and 100.00 mg/L and the concentration series of PI-103 were 0.088, 0.175, 0.35, 0.70 and 1.4 mg/L. After 72 hours treatment, the IC50s of cisplatin for SKOV3 and SKOV3/DDP in the different concentrations of PI-103 were calculated based on the CCK8 assay results.

The cell cycle and apoptosis of SKOV3 and SKOV3/DDP treated with certain concentration the PI-103 and DDP

Based on the IC50 results of DPP for SKOV3 and the inhibition rates of SKOV3/DDP cells treated with PI-103, the appropriate condition, DDP (3.13 mg/L) and PI-103 (0.35 mg/L), was selected to explore the cell cycle and apoptosis of SKOV3 and SKOV3/DDP. The two cell lines were treated as following: SKOV3, SKOV3 + cisplatin, SKOV3 + PI-103, SKOV3 + cisplatin + PI-103, SKOV3/ DDP, SKOV3/DDP + cisplatin, SKOV3/DDP + PI-103 and SKOV3/DDP + cisplatin + PI-103. The cells were digested and single cell suspension was obtained after treated with cisplatin and PI-103 for 72 h, and 1 mL cell suspension (including 1×10⁶ cells) was shifted to the 2 mL EP tubes. The cells were washed 3 times by pre-cooling PBS and then were fixed in pre-cooling 70% ethanol for 24 h.
The tubes were centrifuged at 1,000 g for 3-5 minutes and the supernatant was removed carefully. The precipitation cells were washed by pre-cooling PBS again, and then the propidium (PI) staining solution was added into the tubes. The cells were tested by flow cytometry (488 nm) after incubation in 37 °C for 30 m in dark environment.

Western blot

Cells were treated as described above, and western blot was performed after 72 hours treatment. The cells were washed with ice-cold PBS, and lysis buffer were added to the cells with mixing and shocking on the ice, which made the cells fully cracked and total protein were extracted after centrifugation at 12,000 g for 20 minutes. The protein concentration was determined by the BCA kit. A 15 mg sample of protein was subjected to SDS-PAGE (12% gels). After electrophoresis, proteins of SDS-PAGE were transferred onto PDVF membranes in transfer buffer [25 mM Tris-HCl (pH 7.4) containing 192 mM glycine and 20% v/v methanol] using semidry transfer way (152 mA, 45 m). Membrane were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween20 (TBST) at room temperature for 1 h and then incubated overnight at 4 °C with specific primary antibodies. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL chemiluminescent system (GE Healthcare, Munich, Germany) and exposed X-ray films. Relative optical densities and areas of bands were quantified using an image densitometer. The densitometric plots of the results were normalized to the intensity of the β-actin band.

Statistical analysis

Experimental data were presented as mean ± SEM. All statistical analysis was performed with use of the SPSS 13.5. t-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis was applied. A P value of <0.05 was considered as statistically significant.

Results

The inhibition rates of SKOV3 and SKOV3/DDP cells treated with different concentration of PI-103

In order to observe the impact of PI-103 on the cell proliferation of SKOV3 and SKOV3/DDP, the inhibition rates of SKOV3 and SKOV3/DDP cells treated with different concentration of PI-103 at different time were calculated. As shown in Figure 1, the growth inhibition rate increases along with incubation time. PI-103 increases the inhibition rate of SKOV3 and SKOV3/DDP cells in a dose dependent manner at the three studied time points, i.e., 24, 48 and 72 h. The inhibition rates of SKOV3 and SKOV3/DDP cells were markedly increased when the PI-103 concentration >0.35 mg/L. T reated with 0.35 mg/L PI-103, the inhibition rates of SKOV3 were 17.79%, 28.24%, 32.68% at 24, 48, 72 h, and those of SKOV3/DDP were 15.82%, 25.48%, 32.53% at 24, 48, 72 h.

Figure 1 PI-103 inhibits SKOV3 and SKOV3/DDP proliferation. The inhibition rates of SKOV3 and SKOV3/DDP cells treated with PI-103 were assessed using CCK8 assay. The inhibition rate increased with the elevated concentration of PI-103. The growth inhibition rate was obviously increased when the concentration >0.35 mg/L. T reated with 0.35 mg/L PI-103, the inhibition rates of SKOV3 were 17.79%, 28.24%, 32.68% at 24, 48, 72 h, and those of SKOV3/DDP were 15.82%, 25.48%, 32.53% at 24, 48, 72 h.
The IC50 of cisplatin for SKOV3 and SKOV3/DDP cells
The inhibition rates of SKOV3 and SKOV3/DDP cells treated with different concentrations of cisplatin were determined to calculate the IC50. The results revealed that SKOV3 cells were very sensitive to cisplatin and almost all cells died treated with cisplatin 100 mg/L. However, SKOV3/DDP cells had stronger resistance to cisplatin and the inhibition rate was 85.25% treated with cisplatin 100 mg/L. The IC50s of DDP for SKOV3 and SKOV3/DDP cells were 3.31 and 13.96 mg/L, respectively (Figure 2).

The impact of PI-103 on the IC50 of cisplatin for SKOV3 and SKOV3/DDP cells
To explore whether the combination of PI-103 and cisplatin could exert the more inhibitive effects on SKOV3/DDP, SKOV3 and SKOV3/DDP cells were treated with indicated concentrations of PI-103 and cisplatin, and the optical density (OD) was measured to calculate the cell growth inhibition rate and IC50. The inhibition rates of cisplatin for SKOV3 and SKOV3/DDP treated with the different concentration of PI-103 were displayed in (Figure 3A,B). For PI-103 0 mg/L, the IC50s of cisplatin for SKOV3 and SKOV3/DDP cells were 3.13 and 13.95 mg/L. The IC50s of cisplatin for SKOV3 and SKOV3/DDP cells were changed to 1.12 and 3.85 mg/L treated with PI-103 1.4 mg/L (Figure 3C,D). The results showed that the PI-103 could significantly increase the sensitive of SKOV3/DDP cells to cisplatin.

Figure 2 The difference of IC50s of cisplatin for SKOV3 and SKOV3/DDP cells. The IC50s of cisplatin for SKOV3 and SKOV3/DDP cells were assessed by CCK8 assay. The inhibition rate is lower in SKOV3/DDP cells than in SKOV3 cells. The IC50s of DDP for SKOV3 and SKOV3/DDP cells were 3.31 and 13.96 mg/L, respectively.

Figure 3 PI-103 significantly increase the sensitive of SKOV3/DDP cells to cisplatin. The IC50s of cisplatin for SKOV3 and SKOV3/DDP cells treated with different concentration of PI-103 were assessed using CCK8 assay. The growth inhibition rates of SKOV3 and SKOV3/DDP were increased along with the increased concentration of PI-103 (A,B) and the IC50s were decreased (C,D).
Figure 4 PI-103 and cisplatin blocks cell cycle progress and increase apoptosis in SKOV3 and SKOV3/DDP cells. The influence of PI-103 and cisplatin on cell cycle, apoptosis was assessed by flow cytometry. PI-103 could increase the DNA content of G0/G1 in SKOV3 and SKOV3/DDP cells and the combination of PI-103 + cisplatin further increased the DNA content of G0/G1 in SKOV3 and SKOV3/DDP cells (A,B). In addition, the combination PI-103 + DDP could further increase the apoptosis rate (A,C).

**The cell cycle and apoptosis of SKOV3 and SKOV3/DDP cells treated with certain concentration of PI-103 and DDP**

PI-103 and DDP blocks cell cycle progress in SKOV3 and SKOV3/DDP

In this work, DDP (3.13 mg/L) and PI-103 (0.35 mg/L) was applied to study the effects of DDP and PI-103 on cell cycle and apoptosis of SKOV3 and SKOV3/DDP.

The results were shown in Figure 4A,B. The G0/G1 DNA content of SKOV3 cells was significantly higher than that of SKOV3/DDP cells (38.00±1.32 vs. 29.21±3.56, P<0.05), and treated with cisplatin for 72 h, the G0/G1 DNA content was significantly changed and G0/G1 DNA content of SKVO cells was significantly more than that of SKOV3/DDP (55.20±6.85 vs. 31.88±2.39, P<0.05). This suggests that the resistance of SKOV3/DDP may be correlated with decreased DNA content of G0/G1. After treated with PI-103 for 72 h, the G0/G1 DNA content was not significant changed (51.77±4.00 for SKOV3 vs. 52.18±2.80 for SKOV3/DDP, P>0.05), the G0/G1 DNA content was significantly increased compared to not treated (51.77±4.00 for SKOV3 and 52.18±2.80 for SKOV3/DDP...
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found that the activated Akt protein could
similar trend as p-Akt was observed.
were detected in the SKOV3 group,
expression of cyclin-D1 was
significantly in the SKOV3 and SKOV3/DDP cells
DDP cells, and DDP did as well in SKOV3 cells. The
increased the expression of p21 in SKOV3 and SKOV3/DDP cells.

Apoptosis of SKOV3 and SKOV3/DDP cells treated
PI-103 and DDP

The expression of cyclin-D1, p21, cl-caspase-3
Next, the G1/S-specific protein cyclin-D1, the potent
apoptosis protein cleaved caspase-3 were investigated by
expression of cyclin-D1 was displayed in
expression of cyclin-D1 protein in SKOV3 cells and the combination
DDP was not sensitive to cisplatin, the cyclin-D1 expression

Phosphorylation of Akt and rpS6
The expressions of phospho-Akt and phospho-S6 in
SKOV3 and SKOV3/DDP cells were also examined. The
results were shown in Figure 6A,B. The expression of p-Akt
was higher in SKOV3/DDP cells than in SKOV3 cells
expression, while cisplatin could not. The combination of
further decreased the expression of p-Akt
expression was also detected. The activated caspase-3 was
significantly increased in the treatment group except the
SKOV3/DDP + cisplatin group, and the combination
showed better effects on activating caspase-3 in SKOV3 and
SKOV3/DDP + DDP cells (*P<0.05, *P<0.01) (Figure 5C).

Discussion
Chemotherapy is one of the important therapeutic strategies
for ovarian cancer, and platinum-based combination
chemotherapy is most widely applied in clinical practice.
However, the clinical outcome is often not satisfactory
due to drug resistance. Cisplatin is not recommended for
cases developed drug resistance. Therefore, it is currently
an urgent problem to solve platinum resistance and
improve the chemotherapy sensitivity to platinum-based
chemotherapy drugs for the treatment of ovarian cancer.
The drug resistance of ovarian cancer to chemotherapy
is the results of the complex interaction between multi-gene
and multiple factors (7). Previous studies showed that the
multidrug resistance gene 1/P-glycoprotein (MDR1/P-gp),
the hypoxic microenvironment of ovarian cancer (8) as
well as abnormal regulation of apoptosis could induce drug
resistance. PI3K/Akt/mTOR pathway is involved in the
malignant transformation and chemoresistance (9,10).
Fraser et al. found that the activated Akt protein could
induce the ovarian cancer drug resistance through negative
regulation of PS3 (11). PI3K inhibitor could improve the
ovarian cancer sensitivity to cisplatin by increasing the
mitochondrial Bax translocation and cytC release (12).
Some studies also demonstrated the correlation of
phosphorylation of mTOR with drug resistance. Mabuchi
et al. reported that the mTOR phosphorylation levels was
Figure 5 PI-103 could inhibit the expression of cyclin D1 and increase the expression of p21, cl-caspase-3 in SKOV3/DDP. The protein level of cyclin D1, p21 and cleaved caspase-3 in SKOV3 and SKOV3/DDP cells treated with or without PI-103 and DDP were detected by western blot. PI-103 significantly decreased the protein level of cyclin-D1 and increased the level of p21, cleaved caspase-3 in SKOV3 and SKOV3/DDP cells, and cisplatin did the same in SKOV3/DDP. The combination showed better effects in SKOV3 and SKOV3/DDP cells (*P<0.05, #P<0.01).

higher in cisplatin-resistant ovarian cancer cell line than in cisplatin-sensitive cell lines, and the former is more sensitive to the mTOR inhibitor RAD001 (13), which suggests that the higher mTOR phosphorylation is involved in cisplatin resistance. The activation of the Akt/mTOR pathway can inhibit cisplatin-induced apoptosis of ovarian cancer cells, causing cisplatin resistance in ovarian cancer cells (14).

In this work, we firstly explore the effect of PI-103 on the proliferation of SKOV3 and SKOV3/DDP cells, and the results showed that there was no obvious difference between the two cells. Based on the proliferation curve, 0.35 mg/L was selected as the subsequent concentration. In addition, we found that the IC50 of cisplatin for SKOV3 and SKOV3/DDP cells was 3.31 and 13.96 mg/L, which revealed the strong drug resistance of SKOV3/DDP cells to cisplatin. In the next step, the combination of PI-103 + cisplatin was introduced to explore the inhibition effect. The results showed that the PI-103 could significantly increase the sensitivity of SKOV3/DDP to cisplatin.

Previous studies had demonstrated an association between cell cycle regulation and tumor development, and blockage of the cell cycle progression has become an appropriate target therapy (15,16). Our results indicated that PI-103 could significantly increase the G0/G1 DNA content in SKOV3 and SKOV3/DDP cells and the apoptosis rate treated with cisplatin. The expression levels of cell cyclic proteins and apoptosis proteins were also changed. This suggests that PI-103 could reduce drug resistance of ovarian cancer cell SKOV3/DDP through inhibiting cell proliferation and inducing apoptosis, and PI-103 could enhance the sensitivity of SKOV3/DDP to the cisplatin. PI-103 may be an emerging paradigm for the treatment of cisplatin-resistant ovarian cancer cases.

Treated with PI-103 and cisplatin, the cell cycle was changed. Hence, a question is that whether the proteins in the PI3K/Akt/mTOR signal pathway were also changed. Our results indicated that PI-103 could inhibit the activated Akt and rpS6 but cisplatin could not. However, the combination of PI-103 and cisplatin showed stronger ability of inhibiting the activated Akt and rpS6 either in SKOV3 or SKOV3/DDP cells. This suggests that the interaction between PI-103 and cisplatin could increase the ability of
inhibiting activated Akt and rpS6 and inhibit the PI3K/ Akt/mTOR signal pathway, which is a very important signaling pathway in apoptosis and ovarian cancer (17,18). Schwab et al. reported that PI-103 inhibits the chordoma cell proliferation and promotes apoptosis (19). Chen et al. found that PI-103 could significantly increase the sensitivity of malignant glioma to radiotherapy (20). Many in vitro studies showed that PI-103 also improves the anti-tumor effect of some chemotherapy drugs such as cisplatin, paclitaxel, vincristine, doxorubicin (21).

Tumor cells could dynamically adapt to chemotherapy drugs through complex signaling networks, and this results in drug resistance. Blocking one of such pathways might increase the sensitivity of tumor cells to chemotherapy. Nowadays, it is becoming increasingly apparent that the effects of mostly applied target therapies are limited due to drug resistance. Our results revealed that the dual inhibitor PI-103 could enhance the sensitivity of SKOV3/DDP ovarian cancer cell line to cisplatin, which maybe through inhibiting the activated PI3K/Akt/mTOR signal pathway and increasing the G0/G1 DNA content. Based on this, PI-103 could be the potential candidate to solve drug resistance for ovarian cancer cases. Our results suggest that concomitant inhibition of PI3K and mTOR could be a promising therapeutic strategy for the treatment of ovarian cancer cases developed—drug resistant to cisplatin. However, the detailed and accurate mechanism still needs to be studied in future.

Conclusions

In this research, we explore possible mechanism of enhancing the sensitivity of SKOV3/DDP ovarian cancer cell line to cisplatin chemotherapy by treating with PI-103, a dual inhibitor of phosphatidylinositol 3-kinase and mTOR. The results showed that PI-103 could significantly increase the sensitivity of SKVO3/DDP cells to cisplatin through inhibiting the activation of PI3K/Akt/mTOR signaling pathway and the dual inhibit effect could be a promising therapeutic strategy for the treatment of ovarian cancer cases.
cancer cases developed—drug resistant to cisplatin.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (30973475), the Natural Science Foundation of Jiangsu Province (BK2012749) and the Maternal and Child Health Research Project of Jiangsu Province (F201351).

Disclosure: The authors declare no conflict of interest.

References
