RESEARCH ARTICLE

AZD1480 Can Inhibit the Biological Behavior of Ovarian Cancer SKOV3 Cells *in vitro*

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Abstract

    <u>Objective</u>: To study the mechanism of effects of AZD1480 on the SKOV3 ovarian cancer cell line. <u>Methods</u>: The MTT method was used to assess cellular proliferation, flow cytometry for cellular apoptosis, the scratch test to determine migration, transwell chamber assays to detect cellular invasion, plate clone experiments to detect the clone forming ability and Western blotting to determine p-STAT3 protein levels. <u>Results</u>: The proliferation rate, migration ability, invasiveness and the clone forming ability of SKOV3 cells were reduced after treatment with AZD1480, while apoptosis rate and chemotherapeutic susceptibility were increased. After treatment with AZD1480 plus cisplatin, the apoptosis rate increased significantly while the expression level of p-STAT3 protein was decreased. <u>Conclusion</u>: AZD1480 can inhibit the proliferation, invasion, metastasis and clone formation of SKOV3 cells, induce cellulsar apoptosis, increase the chemotherapeutic sensitivity and reduce the expression level of p-STAT3 protein.

Keywords: SKOV3 cells - JAK inhibitor - AZD1480 - biological behavior - p-STAT3

Introduction

    Ovarian cancer is one of the malignant tumors of female, epithelial ovarian cancer (EOC) is relatively common one. It has the features of occult, easy to metastasis and invasion, the chemotherapy drug tolerance and poor prognostic. Treatment of EOC was mainly by operation, combined with chemotherapy. With the research of cell signal transduction pathways, blocking the cell signal transduction pathway may be a new direction for therapy of malignant tumor.

    JAK2 is a kind of cytoplasmic protein tyrosine kinase (PTK), JAK plays an important role in signal transduction of a variety of cytokines. The signal transducer and activator of transcription (STATs) in signal transduction pathways have multiple family members, in which STAT3 is very important. It was detected to increase abnormally in many solid tumors and participated in malignant tumor cell proliferation, differentiation, invasion, migration and immune escape process (Subramaniam, et al ., 2013; Swiatek-Machado, et al ., 2013). It was proved *in vitro* and *in vivo* that the sustained activation of JAK/STAT3 signal transduction pathway in tissues of head and neck cancer, prostate cancer, breast cancer, colon cancer and other malignant tumors affected the evolution process of biological behavior of tumor cells (Jing et al., 2004; Shin et al., 2009; Lai et al., 2010; Shodeinde et al., 2012). A number of studies have found that there

was overexpression of STAT3 gene in ovarian cancer, persisting of its active form p-STAT3 was closely related with the regulation of proliferation, apoptosis, migration and invasion of ovarian cancer cells (Lai et al., 2010; Kandala et al., 2012).

    AZD1480 is a specific antagonist of JAK2 and can block the activation of STAT3 protein, then regulate the transcription of downstream target gene. It shows the antitumor effects *in vivo* and *in vitro* experiments (Guo et al., 2010; Bid et al., 2012; Couto et al., 2012; Ptak et al., 2012; Yang et al., 2012). However, there was a few data about the effect of AZD1480 on the biological behavior of ovarian cancer cells, so we explored it in this study.

Materials and Methods

Cell culture and morphological observation

    SKOV3 cells were cultured with RPMI1640 (GIBCO) medium containing 10% fetal bovine serum, under 37°C, 5% CO_2 and saturated humidity conditions. The logarithmic growth phase cells within 10 generation were used to do experiments. SKOV3 cells were treated with 5 μmol/L AZD1480 (the selleck company, USA), the morphological changes of the cells were observed under microscope after 24 h.

Proliferation of cells was tested by MTT method

    The cells were divided into control group (Group

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1), DMSO group (Group 2), 5 μmol/L AZD1480 Group (Group 3) and 10 μmol/L AZD1480 Group (Group 4). Cells in the logarithmic growth phase were digested and the single cell suspension was inoculated into 96 well plates, each well was 200 μL, the concentration of cell was 5×10^5/ml, each group was provided with 3 parallel wells. The cells were treated with the corresponding drugs when the cell fusion was about 80% and were cultured at 37°C with 5% CO_2 for 24 hours. Then each well was added 20 μl four methyl thiazolyl blue MTT (Sigma) whose concentration was 5mg/mL and cultured for 4h. MTT reaction solution was discarded and 150 μl of two dimethyl sulfoxide was added into each well. They were vibrated at room temperature for 10 min, the absorbance (A value) at the wavelength of 490 nm of each well was detected with enzymes labelling instrument. Cell proliferation rate (%) = (A values of experimental group A/A values of the control group) × 100%. The experiment was repeated for 3 times.

In the same way, cells were divided into 4 groups of different cisplatin concentration (0 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml) respectively combined with 4 groups of different AZD1480 concentration (0 μmol/L, 1 μmol/L, 5 μmol/L, 10 μmol/L) and composed of 16 groups. The proliferation of cells was tested with the above MTT method.

The apoptosis rate was detected by flow cytometry.

The cells were divided into 16 groups same as the above. Each group cells were collected after treatment for 24h respectively. The cells were washed with pre-cooling PBS for 2 times. Then cells were resuspended with 500 μl pre-cooling binding buffer and their concentration was adjusted to 5×10^6/ml. 100 μl cell suspension was added in the flow tube, then 5 μl FITC-annexin V and 10 μl PI were added and mixed, incubated at room-temperature in dark for15min. Early apoptotic cell numbers were measured by flow cytometry (Beckman company), 1×10^4/ml cells were detected in each group. The results were analyzed using the CXP software and the experiment was repeated for 3 times.

The ability of migration was detected by erasion trace test

Cells in the logarithmic growth phase of each group were digested and the single cell suspension was inoculated into 6 well plates, each well was 2 ml, the concentration of cell was 7.5×10^6/ml. They were erased with 10μl pipet tips when the cell fusion was around 80% and washed with PBS for 3 times. Then they were cultured with serum free RPMI1640 medium for 24 h. Erasion distance was observed and recorded under microscope. The average scratch healing rate was calculated through the measurement of multiple scratch widths. Scratch healing rate= (0 h scratch width - 24 h scratch width)/0 h scratch width × 100%. The experiment was repeated for 3 times.

The cellular invasion ability was detected by transwell chamber assay

The upper surface of the bottom membrane of Transwell chamber (Corning) was covered with a layer of Matrigel matrix. Cells in the logarithmic growth phase of each group were cultured with serum free RPMI1640 medium for 24 h, then they were digested and the single cell suspension was diluted with serum free medium and the concentration of cell was 2.5×10^5/ml, each well was 200 μl. The lower chamber was added 800 μl RPMI1640 medium containing 30% fetal bovine serum medium and cultured for 24h under 37°C, 5% CO_2 and saturated humidity conditions. The membranes were took out and the Matrigel and ventricular cells were wiped with a cotton swab. After formalin fixing and toluidine blue staining, we counted the cells through membrane under microscope and selected 9 different view randomly. The experiment was repeated for 3 times.

The clone forming ability was detected by plate clone experiment

Cells in the logarithmic growth phase of each group were digested and the single cell suspension was inoculated into 6 well plates, each well was 2ml, the concentration of cell was 5×10^2/ml. They were cultured at 37°C with 5% CO_2 and saturated humidity conditions for 10 days. When visible clone cell clusters with naked eye appeared in culture dish, we terminated culture and discarded supernatant. They were washed with PBS carefully for 2 times. Each well was added into 800 μl methanol and fixed for 30 minutes. Then the fixed solution was discarded and 1ml toluidine blue staining solution was added and stained for 30 minutes. They were washed with water slowly and dried in air. They were counted with image analysis software and calculated the rate of cloning efficiency.

p-STAT3 protein was detected using Western blot method.

The cells were divided into 5 groups (medium control and MDSO control group, 1μmol/L AZD1480, 5 μmol/L AZD1480 and 10 μmol/L AZD1480 group). Cells were collected after treatment for 4h and washed with cold PBS for 3 times. Then the cellular lysis buffer was added and incubated on ice for 30min. The total proteins were extracted after centrifugation. Quantitative protein determination was done with BCA kit in accordance with the kit manual.

Statistical analysis

All statistical tests were analyzed by SPSS16.0 statistical software and $p < 0.05$ was considered statistically significant. Differences among groups of single drug treatment were analyzed with single factor analysis of variance. Differences among groups of combined drug treatment were analyzed with double factor variance analysis.

Results

Morphological observation of cells treated with AZD1480

The cellular morphology was meniscus shrinkage, clear boundary, pseudopodia reducing, cytoplasm aggregating to be particles on the cellular inside membrane and particles partly suspended in culture medium after the treatment of AZD1480.

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Table 1. Results of Cell Survival Rate, Apoptosis Rate and Scratch Healing Ratein Different Groups

Group	Cell survival rate	Cell apoptosis rate	Cell Scratch healing rate
ratecontrol group	1.00±0.00	0.75±0.47	0.489±0.006
DMSO group	0.97±0.01	1.60±0.36	0.484±0.005
5 μmol/L AZD1480 group	0.84±0.02	3.87±0.60	0.142±0.008
10 μmol/LAZD1480 group	0.56±0.01	7.06±0.52	0.059±0.005

Table 2. The Test Results of Cell Proliferation Rate in Different Concentration of AZD1480 Combined with Cisplatin Groups

Group	AZD1480 0μM group	AZD1480 1μM group	AZD1480 5μM group	AZD1480 10μM group
Cisplatin 0 μg/ml group	1	0.96	0.8	0.55
Cisplatin 20 μg/ml group	0.67	0.58	0.49	0.35
Cisplatin 40 μg/ml group	0.56	0.48	0.38	0.3
Cisplatin 80 μg/ml group	0.44	0.39	0.27	0.09

Cell survival rate in different groups

There was no significant difference between control group and DMSO group about the cell survival rate (*P* > 0.05). AZD1480 had significant inhibition effect on the growth of SKOV3 cells and the inhibition effect enhanced with the increase of concentration (*P* < 0.05) (Table 1).

In the AZD1480 combined with cisplatin treatment groups (Table 2), cisplatin could inhibit the proliferation of SKOV3 cells with dose dependent (*P* < 0.05). There was no significant difference between 1μmol/L AZD1480 group and control group (*P* > 0.05) but was statistically significant difference 5μmol/L and 10μmol/L AZD148 groups compared with the control group (*P* < 0.05). The Coefficient of drug interaction (CDI) values were (0.902, 0.914, 0.95, 0.893, 0.848, 0.974, 0.923, 0.767, 0.372) < 1, which confirmed that these two drugs were synergistic. CDI value was 0.372 when the concentration was 80μg/ml cisplatin +10μmol/L AZD1480, which showed that their synergistic effects were very significant.

The apoptosis rate in different groups

There was no significant difference between control group and DMSO group about the cell apoptosis rate (*P* > 0.05). The cell apoptosis rate increased in the AZD1480 groups and the apoptosis rate increased with the increase of concentration (*P* < 0.05) (Table 1).

In the AZD1480 combined with cisplatin treatment groups (Table 3), cisplatin could inhibit the apoptosis rate of SKOV3 cells and was dose-independent (*P* > 0.05). There was no significant difference between 1 μmol/L AZD1480 group and control group (*P* > 0.05) but there was statistically significant difference between 5 μmol/L and 10 μmol/L AZD148 groups and the control group (*P* < 0.05).

The cellular migration ability

There was no significant difference between control group and DMSO group about the cellular migration ability (*P* > 0.05). AZD1480 had significant inhibition effect on the migration ability of SKOV3 cells, the migration ability decreased with the increase of concentration (*P* < 0.05). (Table 1)

Table 3. The Test Results of Cell Apoptosis Rate in Different Concentration of AZD1480 Combined with Cisplatin Groups

Group	AZD1480 0μM group	AZD1480 1μM group	AZD1480 5μM group	AZD1480 10μM group
Cisplatin 0 μg/ml group	0.2	3.6	8.5	11.1
Cisplatin 20 μg/ml group	3.1	6.8	9.6	12.6
Cisplatin 40 μg/ml group	6.9	10.1	13.8	14.3
Cisplatin 80 μg/ml group	8.1	15.2	20.3	4 0 . 4

Table 4. Results of Permeated Cell Number and Clone Clusters Number in Different Groups

Group	Permeated cell number	Clone clusters number
Control group	128±6	37±1
DMSO group	123±2	36±2
5 μmol/L AZD1480 group	75±3	27±2
10 μmol/LAZD1480 group	45±3	17±2

The cellular invasion ability

There was no significant difference between control group and DMSO group about the permeated cell number (*P* > 0.05). AZD1480 had significant inhibition effect on the invasion ability of SKOV3 cells, the invasion ability decreased with the increase of concentration (*P* < 0.05). (Table 4)

The clone forming ability

There was no significant difference between control group and DMSO group about the clone number (*P* > 0.05). AZD1480 had significant inhibition effect on the clone forming ability of SKOV3 cells, the clone forming ability decreased with the increase of concentration (*P* < 0.05) (Table 4).

p-STAT3 protein expression

There was no significant difference between control group and DMSO group about the expression level of p-STAT3 gene. AZD1480 decreased the expression level of p-STAT3 gene.

Discussion

The treatment of EOC was mainly on the basis of cytoreductive surgery combined with cisplatin and paclitaxel chemotherapy, the clinical symptoms of most patients were relieved (Piccart et al., 2003; Alberts et al., 2006), but the tumor of most complete remission patients was prone to recurrent (Thigpen et al., 1993), some patients developed resistance. In addition, chemotherapy drugs had toxic effect on normal cells, which limited their clinical application (Monk et al., 2007). At present, Gene therapy for cancer has been the focus of cancer therapy study. The studies have confirmed that ECO was caused by gene expression imbalance and was related to a variety of genes (Son et al., 2012; Shirali et al., 2013 ; Skirnisdottir et al., 2013). Regulating the expression of genes related with ECO to the dynamic balance will become the future development goal for the treatment of ECO.

In this study, we found that JAK2 inhibitor AZD1480 could inhibit the proliferation, apoptosis, invasion and

migration of SKOV3 cells. AZD1480 could increase the cellular sensitivity to cisplatin and these two drugs were synergistic. These provided the experimental basis for reducing dosage and side effects of cisplatin and improving its efficacy in clinical trials.

It was found in many tumors that the excessive activation of STAT3 could affect the growth, apoptosis, invasion and metastasis of tumor (Bromberg et al., 1999; Leong et al., 2003). Many studies confirmed that micro-dose AZD1480 could inhibit the phosphorylation of JAK2-STAT3 in multiple tumors, the expression of its function protein p-STAT3 decreased obviously, thereby regulate downstream genes, such as cyclinD1, cyclinB, cmyc, Bcl-2, MCL1, survivin, which could promote the apoptosis of tumor cells, inhibit the cellular proliferation, migration and invasion and increase the sensitivity to platinum-based chemotherapy drugs (Hedvat et al.,2009; McFarland et al., 2011; Scuto et al., 2011; de et al., 2012; Loveless et al., 2012).

The results of this study showed that 1μm/L AZD1480 could reduce the p-STAT3 protein in SKOV3 cells significantly. However, other cellular biology behavior changes need much higher doses. The possibilities were that the proliferation of SKOV3 cells may also be regulated by other signal pathways, or the inhibition time of small-dose AZD1480 was short and p-STAT3 could fast recovery. The cellular invasion and migration may be closely related to the JAK-STAT3 pathway which may be the main regulation. The biological behavior of cells were inhibited severely and could not recovery in short-term even if the p-STAT3 recovery quickly.

In a conclusion, AZD1480 could inhibit JAK/STAT3 signal pathway in SKOV3 cells effectively and decrease the expression of p-STAT3 protein, which could change some malignant behavior of tumor cells. It gave us inspiration for the treatment of EOC. However, there are many problems need to be further explored. Such as: Only one drug-resistant cell line of ovarian cancer was used in this experiment without comparison with other ovarian cancer cell lines. So we cannot judge the effect of AZD1480 on ovarian cancer cell comprehensively. In addition, *in vivo* experiments should be done due to the complex metabolic processes *in vivo*, the real effect of drugs need to be further confirmed.

References

Alberts DS, Hannigan EV, Liu PY, et a1 (2006). Randomized trial of adjuvant intraperitoneal alpha-interferon in stage III ovarian cancer patients who have no evidence of disease after primary surgery and chemotherapy: An intergroup study. *Gynecol Oncol*, **100**, 133-8.

Bid HK, Oswald D, Li C, et al (2012). Anti-Angiogenic Activity of a Small Molecule STAT3 Inhibitor LLL12. *PLoS One*, **7**, e35513.

Bromberg JF, Wrzeszczynska MH, Devgan G, et al (1999). STAT3 as an oncogene. *Cell*, **98**, 295-303.

Couto JP, Almeida A, Daly L, et al (2012). AZD1480 Blocks Growth and Tumorigenesis of RET- Activated Thyroid Cancer Cell Lines. *PLoS One*, **7**, e46869.

de Groot J, Liang J, Kong LY, et al (2012). Modulating Antiangiogenic Resistance by Inhibiting the Signal Transducer and Activator of Transcription 3 Pathway in Glioblastoma. *Oncotarget*, **3**, 1036-48.

Guo W, Wu S, Wang L, et al (2011). Antitumor activity of a novel oncrasin analogue is mediated by JNK activation and STAT3 inhibition. *PLoS One*, **6**, e28487.

Hedvat M, Huszar D, Herrmann A, et al (2009). The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell*, **16**, 487-97.

Jing N, Li Y, Xiong W, et al (2004). G-quartet oligonucleotides: a new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. *Cancer Res*, **64**, 6603-9.

Kandala PK, Srivastava SK (2012). Regulation of Janus-activated kinase-2 (JAK2) by diindolylmethane in ovarian cancer *in vitro* and *in vivo*. *Drug Discov Ther*, **6**, 94-101.

Lai SY, Johnson FM (2010). Defining the role of the JAK-STAT pathway in head and neck and thoracic malignancies: implications for future therapeutic approaches. *Clini Drug Resist Updat*, **13**, 67-78.

Leong P L, Andrews G A, Johnson D E, et a1(2003). Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. *Proc Natl Acad Sci USA*, **100**, 4138-43.

Loveless ME, Lawson D, Collins M, et al (2012). Comparisons of the efficacy of a Jak1/2 inhibitor (AZD1480) with a VEGF signaling inhibitor (cediranib) and sham treatments in mouse tumors using DCE-MRI, DW-MRI, and histology. *Neoplasia*, **14**, 54-64.

McFarland BC, Ma JY, Langford CP, et al (2011). Therapeutic potential of AZD1480 for the treatment of human glioblastoma. *Mol Cancer Ther*, **10**, 2384-93.

Monk BJ, Tewari KS, Koh WJ(2007). Multimodality therapy for locally advanced cervical carcinoma: state of the art and future directions. *J Clin Oncol*, **25**, 2952-65.

Piccart MJ, Bertelsen K, Stuart G, et a1 (2003). Long-term follow-up confirms a survival advantage of the paclitaxel-cisplatin regimen over the cyclophosphamide-cisplatin combination in advanced ovarian cancer. *Int J Gynecol Cancer*, **13**,144-8.

Ptak A, Gregoraszczuk EL (2012). Bisphenol A induces leptin receptor expression, creating more binding sites for leptin, and activates theJAK/Stat, MAPK/ERK and PI3K/Akt signalling pathways in human ovarian cancer cell. *Toxicol Lett*, **210**, 332-7.

Scuto A, Krejci P, Popplewell L, et al (2011). The novel JAK inhibitor AZD1480 blocks STAT3 and FGFR3 signaling, resulting in suppression of human myeloma cell growth and survival. *Leukemia*, **25**, 538-50

Shin DS, Kim HN, Shin KD, et al (2009). Cryptotanshinone inhibits constitutive signal transducer and activator of transcription 3 function through blocking the dimerization in DU145 prostate cancer cells. *Cancer Res*, **69**, 193-202.

Shirali S, Aghaei M, Shabani M, et al (2013). Adenosine induces cell cycle arrest and apoptosis via cyclinD1/Cdk4 and Bcl-2/ Bax pathways in humanovarian cancer cell line OVCAR-3. *Tumour Biol*, **34**, 1085-95.

Shodeinde AL, Barton BE (2012). Potential use of STAT3 inhibitors in targeted prostate cancer therapy: future prospects. *Onco Targets Ther*, **5**, 119-25.

Skirnisdottir I, Seidal T (2013). Association of p21, p21 p27 and p21 p53 status to histological subtypes and prognosis in low-stage epithelial ovarian cancer. *Cancer Genomics Proteomics*, **10**, 27-34.

Son DS, Kabir SM, Dong YL, et al (2012). Inhibitory effect of tumor suppressor p53 on proinflammatory chemokine expression in ovarian cancer cells by reducing proteasomal

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degradation of IκB. *PLoS One*, **7**, e51116.

Subramaniam A, Shanmugam MK, Perumal E, et al (2013). Potential role of signal transducer and activator of transcription (STAT) 3 signaling pathway in inflammation, survival, proliferation and invasion of hepatocellular carcinoma. *Biochim Biophys Acta*, **1835**, 46-60.

Swiatek-Machado K, Kaminska B (2013). STAT signaling in glioma cells. *Adv Exp Med Biol*, **986**, 189-208.

Thigpen JT, Vance RB, Khansur T (1993). Second-line chemotherapy for recurrent carcinoma of the ovary. *Cancer*, **71**, 1559-64.

Wang X, Crowe PJ, Goldstein D, et al (2012). STAT3 inhibition, a novel approach to enhancing targeted therapy in human cancers (Review). *Int J Oncol*, **41**, 1181-91.

Yang CL, Liu YY, Ma YG, et al (2012). Curcumin blocks small cell lung cancer cells migration, invasion, angiogenesis, cell cycle and neoplasia through Janus kinase-STAT3 signalling pathway. *PLoS One*,**7**, e37960.