Synthetic paclitaxel-octreotide conjugate reverses the resistance of paclitaxel in A2780/Taxol ovarian cancer cell line

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Abstract. The high mortality of ovarian cancer is partly due to the frequent resistance of ovarian cancer to current chemotherapy agents such as paclitaxel and platinum. Somatostatin analogue (SSTA) has been shown to inhibit the proliferation of some tumors through binding to somatostatin receptor (SSTR) and activation of Ras-, Rapl- and B-Raf-dependent extracellular signal-regulated kinase 2 (Erk2). It was reported that paclitaxel-octreotide conjugate (POC) exhibited enhanced tumor growth inhibition with reduced toxicity. In the present study, we prepared the POC and investigated its effects and mechanism for the reversal of drug resistance in paclitaxel-resistant ovarian cancer cell line. We demonstrated that treatment with POC led to more cell apoptosis than either paclitaxel or octreotide (OCT) alone. Moreover, the expression of multidrug resistance 1 (MDR1) and vascular endothelial growth factor (VEGF) mRNA, and protein decreased in a dose-dependent manner while the expression of SSTR remained stable following treatment with POC. Although the exact action, in vivo effects and pharmacologic kinetics of POC remain to be investigated, we have demonstrated the feasibility for the synthesis of POC, and more significantly, provided a potential approach to overcome the resistance of ovarian cancer against taxol. The findings also shed some new light on the mechanisms underlying the development of resistance to taxol by ovarian cancer cells.

Introduction

Ovarian cancer is the most severe gynecologic malignancy, causing 114,000 deaths a year globally. In the USA alone, an estimated 23,000 women are diagnosed with ovarian cancer each year and the 5-year survival rate is merely 30% (1). In the United States, ovarian cancer represents 3% of all the new cancer cases in women, and accounts for 5% of all the cancer deaths (2). The high mortality is partly due to the frequent resistance of ovarian cancer to chemotherapy regimens. Paclitaxel combined with platinum remains to be the first line chemotherapy for ovarian cancer. Paclitaxel is a small molecule cytotoxin targeting tubulin and has strong cytostatic and apoptotic effects. Unfortunately, while most patients initially respond to this combined chemotherapy, the majority of these (up to 75%) will eventually relapse within 18 months with many having drug resistant disease (3). Ovarian cancer cells develop drug resistance through different pathways depending on the drug used (4). Multiple mechanisms can mediate the development of paclitaxel resistance, including changes in: i) the regulation or repair of the primary target of the drug (DNA, microtubule); ii) drug retention (increased efflux or decreased uptake); iii) drug inactivation or sequestration; and iv) signaling pathways that affect cell cycle/apoptosis. Paclitaxel is known to be transported by the ATP-dependent efflux pump P-glycoprotein (multidrug resistance, MDR) and upregulation of MDR has been associated with clinical drug resistance to various agents (5,6).

There is an imperative need for the development of new treatment modalities to improve the management of ovarian cancer patients. Switch to alternative drugs with different therapeutic mechanisms is one strategy to overcome the resistance against the presently used drugs. However, limited success has been achieved with the use of second line chemotherapy following the recurrence of ovarian cancer or the resistance to the first
line drugs (7). This failure is often caused by the activation of ‘generic’ resistance mechanism against multiple drugs sharing a specific feature. Rationalized design and targeted chemotherapy using modified drugs equipped with new features to avoid the resistance of cancer cells may potentially enhance the drug efficacy and reduce the toxicity of cancer therapies.

SST is a cyclic polypeptide hormone that is found in most human organs and tissues. SST has a broad range of cellular functions such as inhibition of secretion and blocking of cell proliferation and cell survival (8,9). Natural somatostatin (SST) has limited clinical applications because of its low selectivity and short half-life. However, somatostatin analogue (SSTA) is widely applied and has been shown to have more powerful effects and a longer half-life. It has been shown that SSTA is able to inhibit the proliferation of neuroendocrine tumors in vitro as well as tumor growth in vivo (10-14). The specific somatostatin receptor (SSTR), with five subtypes, mediates the functions of SSTA. Two or more receptor subtypes, particularly SSTR2 are often detected in ovarian cancers (15,16) and most types of other tumors (17,18). It is known that SSTR2 mediates the inhibition of cell proliferation via the activation of Ras-, Rapl- and B-Raf-dependent extracellular signal-regulated kinase 2 (Erk2) (11,12,19). Octreotide (OCT) is the most widely used SSTA in clinical applications. OCT was found to inhibit the proliferation of SSTR2-expressing cells (20,21). In previous studies, we showed that OCT could inhibit ovarian cancer cell proliferation and showed that OCT could inhibit ovarian cancer cell proliferation and cell survival (8,9). The mechanisms by which SST and SSTA enhance the antitumor activity of paclitaxel rely on its capability of promoting tubulin assembly into microtubules and the resultant interference with the G2-M transition of cell cycle (24,25).

Materials and methods

The synthesis of POC. Direct synthesis of OCT acetic acid and paclitaxel succinic acid derivatives was prepared for target products. Paclitaxel of 200 mg and succinic anhydride of 300 mg were dried in vacuum for 5 h, dissolved in 5 ml dry pyridine, and mixed for reaction at 30°C for 24 h. The reaction products were re-dissolved in 10 ml of acetone and the paclitaxel succinyl anhydride was extracted from solid precipitation in conditions of drying and reduced pressure, followed by adding and stirring with 10 ml of water dropwise. Paclitaxel succinyl anhydride of 25 mg, SDPP (N-hydroxysuccinimido diphenyl phosphate) of 30 mg and triethylamine of 30 mg were dissolved in 0.5 ml anhydrous acetonitrile with stirring overnight at room temperature. The preliminary product mixture was followed by vacuum concentration process and then re-dissolved in ethyl acetate. Finally, the target product was successfully recovered by washing and drying process.

Cell culture. Human ovarian cancer cell line A2780 (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) and A2780/Taxol (Bogoo Biotechnology, Co., Ltd., Shanghai, China) were cultured at 37°C, 5% CO₂ atmosphere and 90% humidity, in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Waltham, MA, USA). The cells were passaged every 2-3 days using 0.25% trypsin (Sigma-Aldrich, Schnelldorf, Germany). The log-phase cells were collected for further experiment.

Confocal microscopy. To evaluate the targeted binding of POC to SSTR2 positive cells, we observed the internalization of fluorescein-labeled POC into A2780/Taxol cells at different times. A2780/Taxol cells cultured with fluorescein-labeled POC (10 nmol/ml) were detected by confocal microscope (Olympus Fluoview™ FV1000; Olympus, Tokyo, Japan) at 30 min, 2 h and 8 h when after preparation of phosphate-buffered saline (PBS) buffer washing 3 times.

Cell proliferation assay. A2780/Taxol cells (Bogoo Biotechnology) in the log-phase were seeded in each well of the 96-well culture plates and cultured at 37°C under a 5% CO₂ atmosphere for 24 h. The cells were incubated in 100 µl of medium with paclitaxel (0, 1.25, 2.5, 5, 10 and 20 nmol/ml), OCT (0, 1.25, 2.5, 5, 10 and 20 nmol/ml), or POC (0, 1.25, 2.5, 5, 10 and 20 nmol/ml). At different time-points, cells were treated with 10 µl of the Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto Japan) for 3 h. Absorbance (A) was measured on an enzyme-linked immunosorbsent assay plate reader. The inhibition rate was calculated using the following formula: Cell proliferation inhibition rate = (average of value A from the control group - the average of value A from the experimental group)/(average of value A from the control group - average of value A from blank controller) x 100%. Resistance index was calculated with the following formula: IC50 of resistant A2780 cells/IC50 of parental A2780 cells. All experiments were repeated in triplicate and more than three wells were used for each treatment.

Detection of cell apoptosis. The experiment contained four groups comprising the control, paclitaxel (10 nmol/ml), OCT (10 nmol/ml) and POC (10 nmol/ml). Following treatment for 36 h, cell apoptosis was examined using the Annexin V-FITC/PI staining kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing,
Table I. Primer sequences for the real-time PCR reaction.

<table>
<thead>
<tr>
<th>Objective gene</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>β-actin-Forward</td>
<td>GATGACCCAGATCATGTTTGAG</td>
</tr>
<tr>
<td>β-actin-Reverse</td>
<td>AGGGCATAACCCTCGTAGAT</td>
</tr>
<tr>
<td>SSTR2-Forward</td>
<td>CATTTATGTCATCTCCGCTAT</td>
</tr>
<tr>
<td>SSTR2-Reverse</td>
<td>TGATTGATGCCATCCACAGT</td>
</tr>
<tr>
<td>VEGF-Forward</td>
<td>CAGAAGGGAGGCCAGAAT</td>
</tr>
<tr>
<td>VEGF-Reverse</td>
<td>CACAGATGCCATCCAGATG</td>
</tr>
<tr>
<td>MDR1-Forward</td>
<td>GTGTCAGGTGCCATCAAT</td>
</tr>
<tr>
<td>MDR1-Reverse</td>
<td>TGGAAAGGAGCGGTGTAA</td>
</tr>
</tbody>
</table>

Immunocytochemistry. The cells cultured on coverslips in 6-well plates were fixed in 4% paraformaldehyde for 30 min, washed with PBS for 5 min and permeabilized with Triton X-100 (Sigma-Aldrich). The cells were incubated with 10% goat serum for 20 min for blocking. Primary rabbit monoclonal antibody (anti-SSTR2, 1:100; Abcam) was added and the incubation continued overnight at 4°C in a humidified chamber. After washing with PBS, HRP-labeled secondary antibody was applied for 30-min incubation. Coverslips were immersed in freshly prepared DAB solution (Dako Denmark A/S, Glostrup, Denmark) for color development. Cells were counterstained with hematoxylin for 10 min and microscopic observation was performed for the detection of SSTR2 expression.

Real-time PCR. Total RNA was extracted from A2780/Taxol cells treated by conjugate for 48 h with the use of TRizol reagents. RNA concentration was measured on a UV spectrophotometer based on the absorbance values at 260 and 280 nm. cDNA was synthesized using 1 µg of total RNA according to the instructions provided in the RT-PCR kit (Takara). Designation and sequences of PCR primers (Houzai Co., Tokyo, China) are provided in Table I. Real-time PCR was performed in a LightCycler (Roche Applied Science) under the following conditions: pre-80 denaturation at 94°C for 2 min, then denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 45 sec. Fold of difference relative to the reference gene (β-actin) was determined by conversion of 2^−ΔΔCT. ΔΔCT = (CTobjective gene − CTreference gene) of experimental group − (CTobjective gene − CTreference gene) of control group.

Western blot analysis. Following our previously established method (29), A2780/Taxol cells treated by conjugate for 48 h were lysed using modified RIPA lysis buffer (1% NP-40, 0.25% deoxycholic acid, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 g/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 2 g/ml pepstatin and 1 g/ml aprotinin). Cell lysates were boiled in the loading buffer (3.3% glycerin, 1% SDS, 20 mM TRIS, pH 6.8, 23 mM β-mercaptoethanol freshly added and 0.4 mg/ml bromophenol blue). Proteins were separated in precast gradient SDS-PAGE (4-20%) and transferred to polyvinylidenefluorid (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA), and then incubated with specific primary antibodies for 2 h at room temperature, followed by 1 h of incubation with appropriate HRP-conjugated secondary antibodies. Western blot analyses were performed by using primary antibodies against SSTR2 (1:1,000), IGF-1 (1:600), VEGF (1:1,000). The antigen-antibody complexes in western blot analysis were detected with an enhanced chemiluminescence detection system (Amersham Biosciences, Pittsburgh, PA, USA). Specific protein bands were visualized after autoradiography. The intensity of each protein band was quantified by using image analysis software and normalized against corresponding β-actin that was detected by anti-β-actin antibody (1:2,000).

Statistical analysis. Statistical analysis was performed using the SPSS 16.0 software. Data are expressed as the means ± SD. The ANOVA and Student’s t-test was used for comparison of the drug-treated and drug-untreated controls. A P<0.05 was considered to indicate a statistically significant result.

Results

Synthesis of POC. Direct synthesis of OCT acetic acid and paclitaxel succinic acid derivatives was performed as described in Materials and methods. As shown in Fig. 1, the process consisted of sequential synthesis of the following four chemicals: i) N-hydroxysuccinimido diphenyl phosphate; ii) paclitaxel succinyl anhydride; iii) N-hydroxy paclitaxel succinyl anhydride; and iv) POC.

SSTR2 expression in A2780/Taxol cells. In order to confirm the SSTR2 expression in the cell model, we performed immunocytochemistry using the specific antibody against SSTR2. As shown in Fig. 2, A2780/Taxol cell membranes display strong positive staining.

Internalization of fluorescein-labeled POC in A2780/Taxol cells. The specific fluorescein were visible mainly along the cell surface of the A2780/Taxol cells at 30 min of culture, whereas part of the denser fluorescent grains appeared into the cytoplasm at 2 h. Moreover, fluorescent grains were visibly distributed through the cytoplasm, and many grains were concentrated around cell nucleus at 8 h (Fig. 3). These results strongly suggested that POC has a favorable and specific binding targeted to SSTR2-positive cells, which resulted from the SSTR2-mediated internalization in A2780/Taxol cells.

Effect of POC on A2780/Taxol cell proliferation. The A2780/Taxol cells were round, cytoplasm-rich and grew vigorously under normal conditions. Without treatment, cells were transparent, spread evenly, and with smooth and complete edges and had similar sizes and shapes. Following POC treatment for 24 h, the number of adherent normal cells decreased, and the cell-cell space became larger. Some cells appear to be condensed, darker and displayed morphological features of apoptosis including shrinkage, foaming and formation of apoptotic bodies.
As the treatment time prolonged, cell growth became slow and there were increased numbers of floating cells and cell debris. According to the dose-effect curves of paclitaxel on A2780/Taxol and A2780 cells, the calculated resistance index (RI) of A2780/Taxol cell to paclitaxel was 28.33. POC exhibited an enhanced inhibitory effect on A2780/Taxol cell proliferation, with the calculated resistance index (RI) reaching 4.2 (Fig. 4). Notably, the POC inhibited proliferation
At the indicated time (24, 48 and 72 h) in a concentration-dependent manner (P<0.05; Table II and Fig. 5).

Enhanced apoptotic effect of POC in A2780/Taxol cells. Compared to the control group, increased apoptosis was observed in paclitaxel (10 nmol/ml), OCT (10 nmol/ml) and POC (10 nmol/ml) groups (P<0.05). Both the effect of POC group and that of OCT group were much more powerful than the paclitaxel group (P<0.05). In addition, the effect of POC group was more powerful than the OCT group (P<0.05) (Fig. 6).

Alteration of SSTR2, MDR1 and VEGF mRNA expression following drug treatment. To investigate the mechanism responsible for the enhanced effects of POC, mRNA was isolated, and SSTR2, MDR1 and VEGF mRNA expression was determined following treatment with POC. As shown in Fig. 7, SSTR2 mRNA was detected in each group, but there is no difference between the various concentrations (P>0.05). Compared to the control group, the expression of both MDR1 and VEGF mRNA decreased in a dose-dependent manner following 48 h of treatment with POC (P<0.05), indicating their involvement in the POC-mediated cell effects.

![Figure 3. Confocal microscopy of fluorescein-labeled POC in A2780/Taxol cells. After 30 min of incubation with 10 nmol/ml fluorescein-labeled POC, fluorescein is mainly located at the membrane of the cells (arrowheads) (A), and upon incubation for 2 h, fluorescent labels appeared in the cytoplasm (arrowhead) (B). Fluorescent grains were visibly distributed through the cytoplasm of the cells and many grains were concentrated around the nucleus (C).](image)

![Figure 4. The dose-effect curves of paclitaxel in A2780 and A2780/Taxol cells. The cell viability was examined by CCK-8 assay. Inhibition concentration (50%) (IC50) of paclitaxel was 0.94 nmol/ml in A2780 cells (A), and 26.56 nmol/ml in A2780/Taxol cells (B), respectively. The calculated resistance index (RI) of A2780/Taxol cell to paclitaxel was 28.33. The altered IC50 and RI reached 3.95 and 4.2 nmol/ml (C), respectively, after treatment with conjugate for 48 h.](image)

![Figure 5. The inhibitory effect of POC in A2780/Taxol cells. Cells were treated with POC at the indicated concentration and time. POC exhibited an enhanced inhibitory effect on A2780/Taxol cell proliferation (24, 48 and 72 h) in a time- and concentration-dependent manner (P<0.05).](image)
MDR1 and VEGF. The intensity of protein bands was read by densitometry and the standardized results with β-tubulin are summarized in Table III. The results indicated that following 48 h of treatment, both proteins were decreased in a dose-dependent manner (P<0.05) (Fig. 8).

**Discussion**

Feasibility and simplicity are key factors to be considered for the design of synthesis strategy. Huang et al (26) firstly reported that paclitaxel succinyl anhydride reacts with OCT from solid phase synthesis through the catalysis of Benzotriazol-1-yl-oxytrypyrrolidino-phosphonium hexafluorophosphate (PyBOP) and POC is obtained after dissociating from resin. However, OCT resin complex in this method is not available in the market and the catalyst PyBOP is expensive which bring us difficulty in synthesis and application of POC. In this study, we adopted a new synthesis approach, which overcomes the difficulty of unavailable OCT resin complex and expensive catalyst. The process included the sequential synthesis of N-hydroxysuccinimido diphenyl phosphate, paclitaxel succinyl anhydride, N-hydroxy succinyl acid ester and POC. Using this method, POC can be synthesized in a large amount from cheap reagent Diphenyl N-succinimid-ster (SDPP). This has opened a new route for mass production by pharmaceutical industry, and thus, opening the door for large scale clinical trials on this bioactive agent.

Paclitaxel combined with platinum remains the first line chemotherapy in the treatment of ovarian cancer. However, due to resistance, it often fails to cure patients. Therefore, the reversal of paclitaxel resistance in ovarian cancer and increased sensitivity to paclitaxel-based chemotherapy drugs is a crucial issue. Our earlier study showed that chemotherapy agent combined with OCT could markedly inhibit the proliferation and promote apoptosis of resistant ovarian cancer cells. This combination of the two single drugs has significant synergistic action (22,23). Furthermore, studies on synthesized POC in lung cancer cells have shown its cytotoxicity in vitro and in vivo (26-28). The thought for increased efficacy of OCT-conjugated taxol was based on the hypothesis that after binding to SSTR, through SSTR endocytosis, OCT-conjugated taxol would internalize into the cytosol of SSTR-expressing tumor cells and therefore increasing the intracellular concentration of paclitaxel. The present study of confocal microscopy also supported this opinion. In addition, this may lead to a decreased toxicity of taxol in non-SSTR-expressing cells (26).

Our current data suggested that POC inhibited A2780/Taxol cell proliferation, increased the chemotherapeutic sensitivity...
of paclitaxel and reversed chemotherapy resistance. Moreover, we found that the mRNA levels of SSTR2 were not altered but the mRNA levels of MDR1 and VEGF were significantly reduced following POC treatment, whereas, the expression of SSTR2, MDR1 and VEGF protein appeared to be decreased by conjugate treatment. The relatively stable levels of SSTR2 mRNA and significant reduction in protein indicated its post-translational regulation. Interestingly, Huang et al (26) reported that short-term OCT treatment could lead to SSTR2 desensitization, resulting in a reduced inhibitory effect on hepatocellular carcinoma cells by OCT. However, long-term OCT treatment effectively inhibited the development and growth of hepatocellular carcinoma cells probably via resensitization and upregulation of SSTR2. This discrepancy for short-term effect on SSTR2 protein levels may be due to the different characteristics of hepatocellular carcinoma cells and ovarian cancer cells, the different expression levels of the receptor, and the higher intracellular concentration that the conjugate may potentially achieve. Nevertheless, our results have pointed to the possibility that POC may exert its effect through decreasing SSTR2 expression.

MDR1 was found to decrease the intracellular paclitaxel concentration, leading to a reduced or loss of drug function in ovarian cancer cells (30,31). This study demonstrated a decreased MDR1 expression in A2780/Taxol cells by the treatment with POC on both mRNA and protein levels, suggesting that MDR1 may be involved in POC-mediated inhibition of cell proliferation and reversal of paclitaxel resistance. Vascular endothelial growth factor-A (VEGF-A, commonly known as VEGF) is a key pro-angiogenic factor that plays a crucial role in tumor expansion (32). Akiyama et al (33) observed that VEGF secreted from tumors upregulated MDR1 through the activation of VEGFR2 and Akt. MDR1 upregulation, via the VEGF-VEGFR pathway in the tumor microenvironment, is one of the mechanisms of drug resistance acquired by tumor endothelial cells. This study demonstrated a decrease of VEGF mRNA and protein expression in A2780/Taxol cells by POC, suggesting that one action of this agent may be through the downregulation of MDR1 and inhibition of VEGF expression. More detailed studies are required to elucidate how the POC could inhibit the expression of VEGF and the downstream genes.

The present study demonstrates the effects of POC on A2780/Taxol cells and explored the possible mechanism mediated by SSTR2, MDR1 and VEGF. The study introduces a potential chemotherapeutic reagent for ovarian cancer therapy. However, the mechanism of the metabolism, transportation and pharmacological dynamics of POC from the extracellular to intracellular departments remain unclear. Future mechanistic and in vivo studies are required for a better understanding of this novel agent.

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References


Table III. Effect of POC on expression of SSTR2, MDR1 and VEGF protein in A2780/Taxol cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>2.5 nmol/ml</th>
<th>5.0 nmol/ml</th>
<th>10.0 nmol/ml</th>
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<tr>
<td>SSTR2</td>
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<td>0.486 0.047a</td>
<td>0.348 0.038ab</td>
<td>0.221 0.033ac</td>
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<tr>
<td>MDR1</td>
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<td>0.367 0.057ab</td>
<td>0.242 0.027ac</td>
</tr>
<tr>
<td>VEGF</td>
<td>1</td>
<td>0.771 0.055a</td>
<td>0.568 0.035ab</td>
<td>0.335 0.030ac</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
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*P<0.05 vs. the control group; ^P<0.05 vs. the 2.5 nmol/ml group; ^P<0.05 vs. the 5 nmol/ml group.


