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THE EFFECT OF THE THROMBOSPONDIN-1 MIMETIC PEPTIDE ABT-898 ON THE CHEMOSENSITIVITY OF CISPLATIN-RESISTANT HUMAN OVARIAN CANCER CELLS

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ABSTRACT

Objectives:
Epithelial ovarian cancer (EOC) is the most lethal form of gynecological cancer in women, with an overall 5-year survival of less than 50%. When detected, treatment typically begins with surgical debulking to remove tumor burden, followed by adjuvant chemotherapy. Women usually respond well to initial treatment, however, disease recurrence with chemoresistance is common. Chemoresistance is one factor that contributes to the low survival rates associated with advanced stage EOC. Discovering new methods of resensitizing chemoresistant cells to the effects of chemotherapy is necessary to improve 5-year survival in chemoresistant disease. ABT-898 is a mimetic peptide of the endogenous glycoprotein, thrombospondin-1, that has promising abilities to resensitize chemoresistant cells of ovarian cancer. We hypothesize that ABT-898 will resensitize chemoresistant human ovarian cancer cells to the effects of carboplatin in vitro.

Materials and Methods:
Chemosensitive human ovarian cancer cells, OV2008, and chemoresistant human ovarian cancer cells, C13, were treated with 100μg/mL carboplatin and 100nM ABT-898, individually or in combination. Ki67 and γ-H2A.X immunofluorescence was utilized to assess cellular proliferation and DNA damage respectively.

Results:
Following combination treatment with carboplatin and ABT-898, there was an increase in DNA damage and decrease in proliferation of chemoresistant C13 cells.

Conclusions:
Decreased viability in chemoresistant cells, as shown by diminished proliferation and accumulated DNA damage, provides evidence of possible resensitization to the toxic effects of carboplatin. The results of this study propose a novel method of resensitizing chemoresistant ovarian cancer cells. Further studies are necessary in order to determine significance.

INTRODUCTION

Epithelial ovarian cancer (EOC) is responsible for 5% of cancer related deaths in females and is commonly referred to as the most lethal gynecological malignancy in women.[1] Five-year survival in early, localized stages can be as high as 92%.[1] Unfortunately, due to ambiguous symptoms and poor screening techniques, approximately 85% of cases are diagnosed in the later stages, after metastasis to local or distant sites.1 Late detection instigates the drop in 5-year survival to an average of 46%.[1]

Treatment for EOC begins with surgical debulking followed by adjuvant chemotherapy, including a platinum-based chemotherapy drug, such as cisplatin or carboplatin, and a taxol-based chemotherapy drug, such as paclitaxel. Platinum-based drugs induce cell death by creating double-stranded DNA breaks in the rapidly proliferating cells, while paclitaxel binds to microtubules, thereby inhibiting cellular division.[2] At the beginning stages of treatment, cancer cells are generally sensitive to the effects of carboplatin and 40-60% of women diagnosed in advanced stages completely respond to initial therapy.[3] Median disease-free survival, however, is only about 18 months due to chemoresistant disease relapse.[3] Despite being sensitive to chemotherapy at the onset of treatment, relapse with chemoresistence results in a 5-year survival of approximately 30%.[3] There are many proposed mechanisms for this chemoresistance, including, but not limited to, up-regulated drug efflux pumps and enhanced DNA repair mechanisms.[4,5] Finding new methods of overcoming these mechanisms and resensitizing chemoresistant cells to the effects of chemotherapy is necessary to improve the unfortunate 5-year survival rate of EOC.

Thrombospondin-1 (TSP-1) is an endogenous extracellular matrix glycoprotein that has potent anti-angiogenic and anti-tumorigenic effects.[6] Despite having anti-tumorigenic abilities, the TSP-1 molecule itself is too large to be used clinically. ABT-898 is a mimetic peptide that is derived from the Type I repeat region of TSP-1 and maintains the majority of the biological functions of the native protein.[7] On its own, ABT-898 has the ability to induce tumor regression and prolong survival in a mouse model of EOC.[7] In addition, similar TSP-1 mimetic peptides have been shown to significantly normalize tumor vasculature by increasing endothelial cell death, resulting in improved perfusion of tumor tissue and enhanced uptake of chemotherapy drugs, when used in combination.[8,9]

In this study, we hypothesize that ABT-898 will resensitize chemoresistant cells to the effects of carboplatin in vitro. In order to test this hypothesis, we treated both chemosensitive and chemoresistant human ovarian cancer cells with a combinational treatment of carboplatin and ABT-898, followed by analyses of cellular proliferation and DNA damage.

MATERIALS AND METHODS

Cell Lines:
The two human ovarian cancer cell lines used in this study were OV2008 and C13 (kindly provided by Dr. Benjamin Tsang; Ottawa Hospital Research Institute, Ottawa, ON). OV2008 cells are chemosensitive and C13 cells are chemoresistant to cisplatin. Both cell lines were grown in Roswell Park Memorial Institute (RPMI, Sigma-Aldrich Canada Ltd., Oakville, ON) media supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Canada Ltd., Oakville, ON) and 1% anti-biotic, antimycotic (ABAM, Life Technologies Inc., Burlington, ON). In preparation for treatment, after cells were allowed to adhere to plates overnight, both cell lines were serum-reduced to 1% FBS for 24 hours before treatment application.

Drugs:
ABT-898 was supplied by Abbott Laboratories (Abbott Park, IL) and carboplatin was purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON). Both ABT-898 and carboplatin were reconstituted in sterile water and further diluted into 1% RPMI media to create the concentrations necessary for treatment dosage.

Dose-Response:
Dose response experiments were conducted using both chemosensitive OV2008 cells and chemoresistant C13 cells. Two 96-well plates were seeded with 7,000 cells per well and allowed to adhere and grow in 10% RPMI media overnight. These cells were then serum reduced to 1% RPMI media for 24 hours. One plate was treated with seven different concentrations of carboplatin (1000µg/mL, 600µg/mL, 300µg/mL, 100µg/mL, 60µg/mL, 10µg/mL, 1µg/mL), while the other plate was treated with five different concentrations of ABT-898 (200nM, 100nM, 50nM, 25nM, 12.5nM). Cells were treated for 24 hours. The WST-1 assay was performed to measure cell viability following treatment.

γ-H2A.X:
Both cell types were plated on individual glass coverslips in 24-well plates. 30,000 cells were seeded into each well and allowed to attach and grow in 10% RPMI media overnight. The cells were then serum-reduced to 1% RPMI media for 24 hours. Treatments were applied for 24 hours on the following groups: 1% RPMI control, 100 µg/mL carboplatin only, 100nM ABT-898 only, and a combination of 100 µg/mL carboplatin and 100nM ABT-898. After the treatment period, cells were fixed in 10% formalin for one hour at room temperature. Once fixed, the coverslips were stained for γ-H2A.X (Abcam, Cambridge, MA). During the staining procedure, the cell membranes were permeabilized with 0.1% Triton X (Sigma-Aldrich Canada Ltd., Oakville, ON) in PBS for fifteen minutes at room temperature, then non-specific staining was blocked with 5% serum for ten minutes at room temperature. The primary antibody was then applied (1/1000) and incubated overnight at 4°C after which the secondary antibody was applied (1/100, Cell signaling Technology, Danvers, MA) and incubated for one hour at room temperature. The cells were counterstained with nuclear stain DAPI for one minute at room temperature. After the DAPI treatment, coverslips were mounted on glass slides with prolong gold.

Ki67:
The method for staining for Ki67 followed that outlined above except the primary antibody (1/200, Abcam, Cambridge, MA) was incubated for 1 hour at room temperature, and the secondary antibody (1/100) was incubated for 2 hours at room temperature.

RESULTS

Dose-response:
A high percentage of C13 cells remained metabolically active after treatments of carboplatin (Figure 1A). OV2008 cells, however, greatly decreased in cell viability after carboplatin treatments. 100µg/mL carboplatin killed approximately 50% of the chemosensitive cells in comparison to the 1% RPMI control, therefore this concentration was chosen as the carboplatin dose for this study (Figure 1B). Different concentrations of ABT-898 in combination with 100µg/mL carboplatin had little variability, however, 100nM ABT-898 showed the greatest difference in cell viability of the OV2008 cells (Figure 1D). Therefore, 100nM ABT-898 was chosen as the dose concentration for this study.

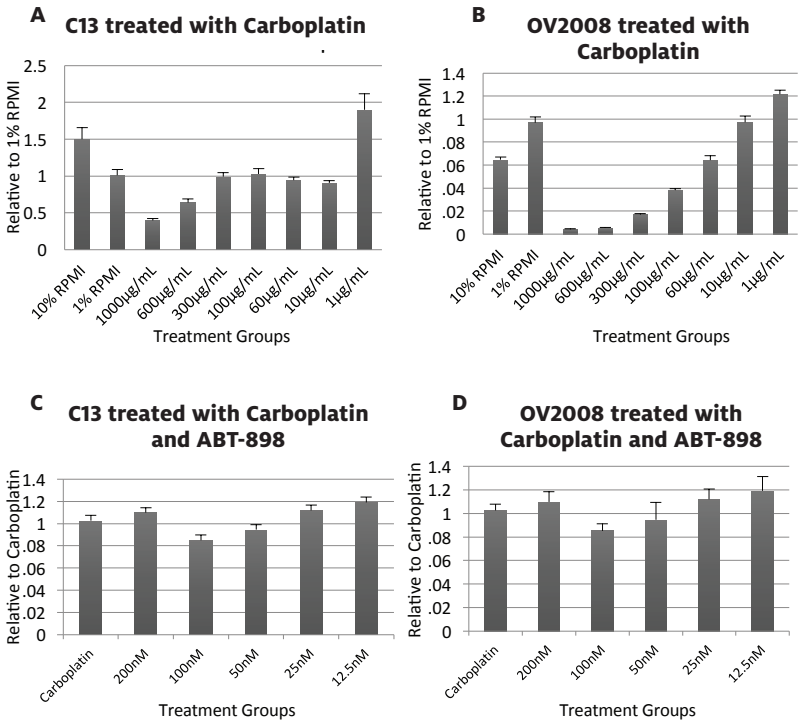

Figure 1: WST-1 assay results. *Treatments with increasing concentrations of carboplatin and ABT-898 to determine ideal drug dose. (A) Carboplatin in C13 cells normalized to 1% RPMI controls. (B) Carboplatin in OV2008 normalized to 1% RPMI controls. (C) 100µg/mL carboplatin and various ABT-898 doses in C13 normalized to 100µg/mL carboplatin control. (D) 100µg/mL carboplatin and various ABT-898 doses normalized to the 100µg/mL carboplatin control.*

γ-H2A.X:
When collecting the results for γ-H2A.X staining, the DAPI counterstain was pseudocolored red and the γ-H2A.X staining was pseudocolored green using the imaging software, MetaMorph. C13 and OV2008 cells in the control groups showed the smallest concentration of γ-H2A.X positive cells visually (Figure 2A). The chemosensitivity of both cell lines are illustrated in the treatment group of carboplatin only. The large number of OV2008 cells lost due to carboplatin treatment greatly contrasted to the small amount of cell loss seen in C13 cells (Figure 2B). ABT-898 only treatments also seemed to have a large impact on DNA damage and ultimately cell death in the chemosensitive cell line (Figure 2C). Combinational treatments of 100nM ABT-898 and 100μg/mL carboplatin displayed the largest concentration of γ-H2A.X positive cells (Figure 2D).

Ki67:
Similar to the γ-H2A.X results, MetaMorph was used to pseudocolor the DAPI counterstain red and pseudocolor the Ki67 stain green. C13 cells treated with a single dose of carboplatin showed the largest concentration of Ki67 positive cells (Figure 3B). However, a single treatment of ABT-898 resulted in the lowest concentration of Ki67 positive cells (Figure 3C).

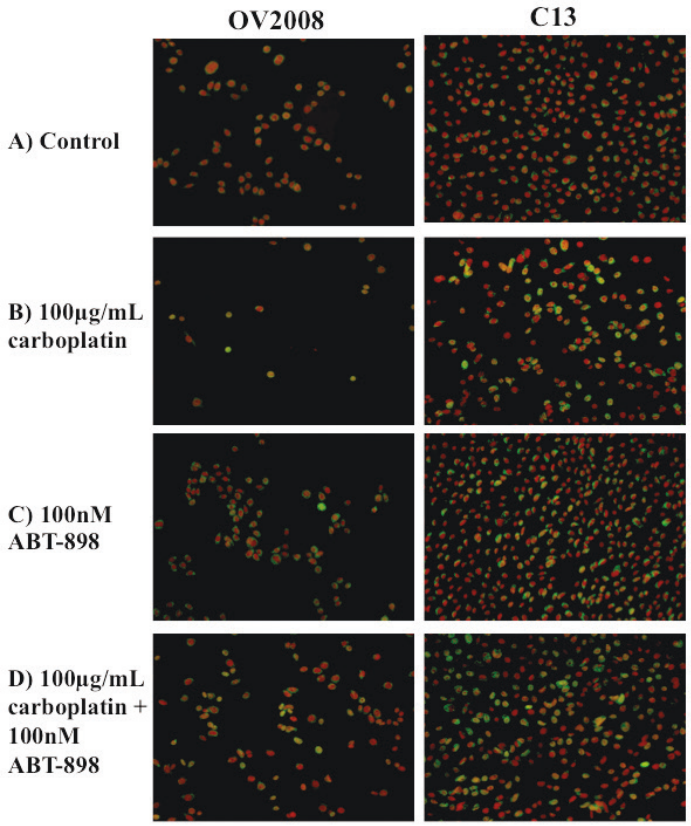

***Figure 2:* γ-H2A.X results.** *DAPI is pseudocolored red and γ-H2A.X is pseudocolored green. Yellow color indicates co-localization of DAPI and γ-H2A.X. (A) 1% RPMI control illustrated lowest concentration of γ-H2A.X positive cells. (B) 100μg/mL carboplatin only. (C) 100nM ABT-898 only. (D) Combination of 100μg/mL carboplatin and 100nM ABT-898 displayed the highest concentration of γ-H2A.X positive cells. (Magnification x 400)*

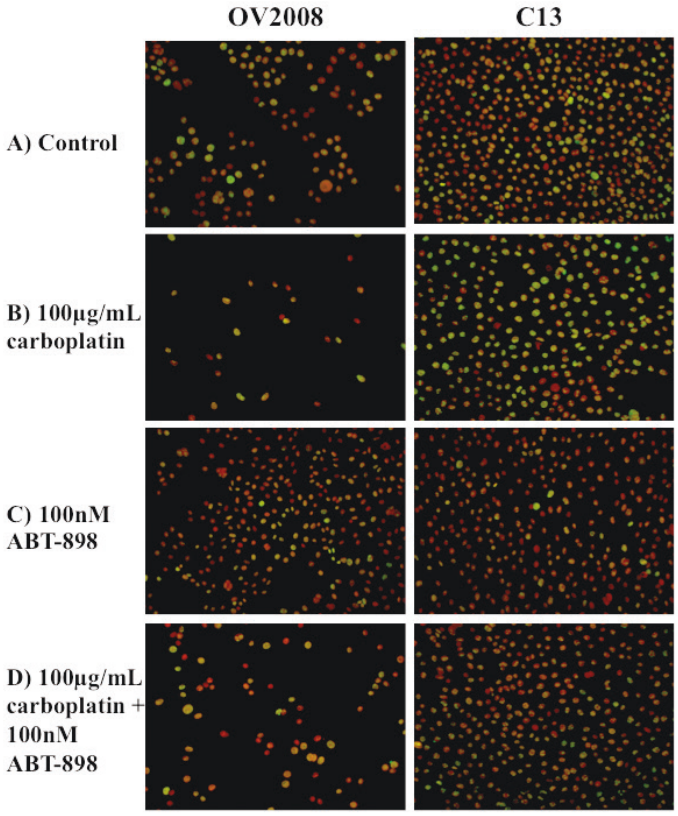

Figure 3: Ki67 results. *DAPI is pseudocolored red and Ki67 is pseudocolored green. Yellow color indicates co-localization of DAPI and Ki67. (A) 1% RPMI control. (B) 100μg/mL carboplatin resulted in the highest concentration of Ki67 positive cells. (C) 100nM ABT-898 produced the lowest concentration of Ki67 positive cells. (D) Combination of 100μg/mL carboplatin and 100nM ABT-898. (Magnification x 400)*

DISCUSSION

Over all, the results of this study suggest that ABT-898 might lead to a decrease in cellular proliferation of chemoresistant C13 cells either alone or in combination with carboplatin. This is illustrated by the distinct decrease in cells displaying the Ki67 marker (Figure 3C & Figure 3D). Furthermore, when used in combination with carboplatin, ABT-898 might result in amplified DNA damage in chemoresistant C13 cells as illustrated by the distinct increase in the γ-H2A.X marker (Figure 2D).

A crucial role of platinum-based chemotherapeutic drugs is to cause double stranded DNA breaks in rapidly proliferating cells.[2] Under ideal circumstances, cancerous cells should not be able to repair such extensive DNA damage, and will therefore initiate cellular apoptosis. There are many theories as to why a cell becomes resistant to chemotherapeutics. One popular belief is that resistant cells acquire the ability to repair these double-stranded breaks and to continue proliferation.[5] This theory is supported by the findings of R.J. Parker and colleagues who reported that resistant cells are twice as efficient at repairing the DNA damage caused by platinum-based drugs than non-resistant cells.[5] Our results illustrated that although carboplatin did cause some DNA damage in the C13 cell line during the 24 hour treatment period, it did not result in nearly as much cell death, and therefore cell loss as seen in the OV2008 cell line (Figure 2B). Despite the mechanism, new methods of overcoming this acquired resistance are necessary for future cancer therapy regimens.

According to previous research, the ABT-898 mimetic peptide seems to have the ability to increase epithelial cell death by approximately 4-fold.[7] One possible mechanism for this is through the increased activation of the Fas/Fas ligand pathway.[10] This pathway ultimately leads to cellular apoptosis through the action of a caspase cascade. There is also evidence to suggest that ABT-898 may significantly increase the delivery and uptake of chemotherapy drugs to EOC tumors by normalizing tumor vasculature.[8,9] Through these mechanisms, it could be possible to resensitize chemoresistant tumors to the effects of chemotherapy. Our experiments were performed in vitro and, therefore, could not replicate the effects of ABT-898 in dynamic endothelial tissue. Although, treatment with ABT-898 alone did result in some increased DNA damage in C13 cells, more damage was seen when it was used in combination with chemotherapy (Figure 2C and Figure 2D). When compared to control groups, the combinational treatment group contains a higher concentration of γ-H2A.X positive cells, correlating to a greater amount of DNA damage. In addition, this evidence supports the idea that ABT-898 enhances the effects of carboplatin because the combination treatment resulted in higher concentrations of double-stranded DNA breaks than seen in the treatment of carboplatin alone (Figure 2B).

Ki67 protein is generally present in cells undergoing proliferation. Therefore, staining for Ki67 immunofluorescence allows for cellular proliferation to be visualized. Increased concentration in Ki67 positive cells implies an amplified amount of cellular proliferation. The evidence from this study suggests that a single treatment of ABT-898 decreases the amount of cellular proliferation in chemoresistant cells when compared to controls and an even greater decrease in proliferation when used in combination with chemotherapeutics (Figure 3C and Figure 3D).

In conclusion, the results from this study provide evidence to suggest that ABT-898 may resensitize chemoresistant cells to the effects of platinum-based chemotherapeutic drugs in vitro. The γ-H2A.X immunofluorescence marker illustrated that combinational therapy of carboplatin and ABT-898 caused a greater concentration of DNA damage in C13 chemoresistant cells when compared to controls. In addition, staining for Ki67 suggested that ABT-898 alone or in combination with chemotherapy could decrease the amount of cellular proliferation occurring in chemoresistant C13 cells. All-in-all, ABT-898 provides a promising solution to aid chemotherapeutic drugs in the targeting of chemoresistant EOC by increasing cellular apoptosis and decreasing proliferation of cancerous cells.

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