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The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells

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ABSTRACT

Objective. Retinoids are important modulators of cell growth, differentiation, and proliferation. 9cUAB30, 9cUAB124, and 9cUAB130 are three novel retinoid compounds that show cytotoxic effects in other malignancies. We evaluated these novel retinoids in combination with chemotherapy against ovarian cancer stem cells (CSCs) *in vitro* and in an *ex vivo* model.

Methods. A2780 cells were plated in 96-well plates and treated with retinoid, carboplatin, or combination therapy. Cell viability was evaluated using ATPLite assay. The A2780 cell line was also analyzed for CSCs by evaluating ALDH activity using flow cytometry. A2780 cells treated *ex vivo* with retinoids and chemotherapy were injected into the flank of athymic nude mice in order to evaluate subsequent tumor initiating capacity.

Results. A2780 cells were sensitive to treatment with retinoids and carboplatin. The best treatment resulted from the combination of retinoid 9cUAB130 and carboplatin. Untreated A2780 cells demonstrated ALDH activity in 3.3% of the cell population. Carboplatin treatment enriched ALDH activity to 27.3%, while 9cUAB130 ± carboplatin maintained the ALDH positive levels similar to untreated controls (2.3% and 6.7%, respectively). Similar results were found in tumorsphere-forming conditions. Flank injections of *ex vivo* treated A2780 cells resulted in 4/4 mice developing tumors at 40 days in the untreated group, while 0/4 tumors developed in the 9cUAB130 and carboplatin treated group.

Conclusion. Combination treatment with carboplatin and retinoids reduced cell-viability, reduced CSC marker expression, and inhibited tumorigenicity, making it a more effective treatment when compared with carboplatin alone.

Introduction

Despite ongoing research efforts and continued search for novel therapeutic agents, ovarian cancer remains the deadliest gynecologic malignancy, with nearly 15,000 women dying of the disease in 2010 [1]. Although most patients initially respond to treatment and achieve a clinical remission following chemotherapy and cytoreductive surgery, nearly 80% die within five years. Since most patients with recurrent ovarian cancer develop chemoresistance, there is a need for new drugs to impact platinum resistant cancer cells. In addition, the prevalence of ovarian CSCs correlates with recurrence in early-stage ovarian cancer [2]. Ongoing areas of research include gene therapy, immunotherapy, targeted therapy, and novel chemotherapeutic agents that not only target the bulk population but also the CSC compartment.

Retinoids are a class of compounds that have been studied for therapy and chemoprevention in numerous malignancies, including ovarian cancer. This class of compounds is comprised of vitamin A, its natural derivatives, and synthetic analogs [3]. Retinoids are known to play an important role in cellular proliferation, differentiation, and apoptosis [4]. Preliminary studies in breast cancer demonstrated that patients receiving a retinoid compound had a significantly decreased risk of developing ovarian cancer. However, follow-up studies showed that this effect was transient and ceased after the retinoid was discontinued [5]. Several synthetic retinoids have been effective against ovarian cancer cell lines and xenografts in animal models [6–13]. Caliaro et al. showed that ovarian cancer cell lines were sensitive to all-trans retinoic acid (ATRA), and that pretreatment with ATRA followed by cisplatin enhanced cytotoxicity compared to cisplatin alone [8]. Likewise, Aebi et al. found that addition of ATRA to cisplatin increased apoptosis in ovarian cancer cell

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lines [7], indicating that the combination of traditional chemotherapy and a retinoid compound may utilize distinct apoptotic mechanisms such that when combined produce additive or synergistic effects. This could result in a promising novel combination treatment strategy. Retinoid therapy has also been postulated to regulate breast CSCs by governing self-renewal and differentiation, and treatment with retinoids (ATRA) potentially induced differentiation thus reducing the CSC pool after treatment [14]. Similarly, ovarian CSC self-renewal and differentiation may also be regulated by retinoid signaling.

A novel retinoid has been developed at the University of Alabama at Birmingham and is currently under investigation in multiple malignancies. 9cUAB30 is a synthetic rexinoid (retinoid × receptor agonist) that has been shown in HL60 leukemia cells to inhibit telomerase and induce apoptosis [15]. 9cUAB30 has also been tested as a chemopreventive agent in a breast cancer animal model and has been approved by the NCI for a chemoprevention clinical trial [16]. The objective of this study was to determine if novel retinoid compounds, specifically 9cUAB30 and two related retinoids, 9cUAB124 and 9cUAB130, can produce enhanced antitumor activity in combination with traditional chemotherapeutic agents in ovarian cancer models. In light of recent developments indicating that ovarian CSCs could be responsible for recurrence and chemoresistance, we investigated whether the retinoids in combination with chemotherapy would produce cytotoxicity against this subpopulation [17,18].

Methods

Cell lines and reagents

A2780 ovarian cancer cells were provided courtesy of Gordon Mills at the University of Texas M.D. Anderson Cancer Center. A2780 cells were grown in RPMI medium supplemented with 10% FBS, 4.5 g/L glucose, 10 mM Hepes buffer, and 1.0 mM sodium pyruvate (Cellgro by Mediatech, Manassas, VA). All cells were maintained at 5% CO_2 atmosphere and 37 °C in antibiotic free media. Carboplatin (Sigma-Aldrich, St. Louis, MO) was prepared as 25 mM stock solution in sterilized water. Three synthetic retinoids (9cUAB30, 9cUAB124, and 9cUAB130) were prepared and provided by Dr. Donald D. Muccio (University of Alabama at Birmingham).

Cell viability assays

A2780 cells were trypsinized and resuspended in culture media. Cells were plated at 1000 cells per well in optically-clear 96-well black plates (Costar #3904, Corning, NY) and incubated at 37 °C for 24 h. Carboplatin and retinoids were diluted to appropriate concentrations in culture medium prior to use. Retinoids were added on days 1, 3, and 5 after plating for dose response assessment; controls were treated with 0.1% DMSO. On day 7, cell viability was determined by measuring cellular ATP levels using an ATPLite luminescence-based assay (Perkin Elmer, Waltham, MA). In the combination treatment studies, retinoid was added on days 1, 3, and 5; carboplatin was added on day 3. The ATPlite assay was performed on day 7. All assays were performed with six replicates in two separate experiments and results are reported as the mean and standard error.

Cancer stem cell marker expression after treatment assay

A2780 cells (single cell suspension) were either plated at 200,000 cells per well in 6-well attachment plates (costar #3904) in standard media, or ultra-low attachment 6-well plates (Costar #3471, Corning, NY) in serum-free EBM-2 (Lonza, Basel, Switzerland) for 24 h prior to treatment. 9cUAB130 was added at a dose of 10 μM on day 1, and carboplatin was added at a dose of 50 μM on day 2 [18]. For attached cells, media was replaced on day 3 and cells were allowed to recover for 24 h. On day 4, cells were harvested, spheres were mechanically dissociated, and attached cells were collected using trypsin. Single cells were counted with visual assessment of viability using trypan blue. The cells plated in low attachment 6-well plates in EBM-2 media were aliquoted (50 μL) into clear bottom 96-well plates to be quantified. Tumorspheres > 50 μm were visually counted using an eye reticle piece by two independent observers. To quantify for expression of the stem cell marker ALDH, an ALDEFLUOR assay (StemCell Technologies, Vancouver, British Columbia) was employed according to the manufacturer's instructions. Cells were suspended in ALDEFLUOR assay buffer with ALDH substrate (at a concentration of 1 μL per 1×10^6 cells) for 45 min at 37 °C. To serve as a control, cells were also resuspended in ALDEFLUOR assay buffer, ALDH substrate, and diethylaminobenzaldehyde (DEAB), which is an inhibitor of ALDH, at a concentration of 1 μL per 1×10^6 cells. All samples were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo.

Ex vivo treatment of A2780 cells and tumor implantation

A2780 ovarian cancer cells were plated in standard media for 24 h and were treated with 9cUAB130 at a dose of 10 μM on day 1. Carboplatin was added at a dose of 50 μM (IC_{80}) on day 2, then media was replaced and cells were incubated for an additional 24 h. Cells were harvested and 200 μL of cells (1:2 cell/matrigel) were injected into the flank of athymic nude mice (four per group). Tumors were monitored once a week and followed until all untreated control mice had a tumor burden that required euthanasia to comply with IACUC regulations (40 days). Tumor size was determined by the product of the two largest diameters.

Results

Cytotoxicity of retinoids and carboplatin in ovarian cancer cell lines

Cell viability assays were performed with three retinoids (9cUAB30, 9cUAB124, and 9cUAB130) in A2780 cells. Treatment with each retinoid yielded an IC_{50} of 5 μM for 9cUAB30, 10 μM for 9cUAB124, and > 10 μM for 9cUAB130 (Fig. 1A). A2780 cells treated with carboplatin yielded an IC_{50} of 15 μM (Fig. 1B). Combination treatment of A2780 cells with each retinoid and carboplatin increased cytotoxicity over retinoid or carboplatin alone (Figs. 2A, B, C). 9cUAB130 was used in tumorsphere-forming and *ex vivo* studies due to its higher cytotoxicity in combination with carboplatin.

Assessment of ALDH enzyme activity

Untreated A2780 cells demonstrated low ALDH positivity (3.3%), and treatment with single agent 9cUAB30 or 9cUAB130 did not significantly alter ALDH positivity (4.9% and 2.3%, respectively) (Table 1). Treatment with single agent carboplatin enriched the population of ALDH positive cells (27.3%) consistent with published literature [18]. Combination treatment with a single dose of 9cUAB30 and carboplatin yielded no significant change in the population of ALDH positive cells (25.7%) as compared to carboplatin alone (27.3%). Combination treatment with a single dose of 9cUAB130 and carboplatin yielded a lower population of ALDH positive cells (6.7%) as compared to carboplatin alone. These results suggest that 9cUAB30 did not alter the CSC population, but the combination of 9cUAB130 and carboplatin was not only cytotoxic, but also did not result in enrichment of CSCs, indicating the combination treatment targeted the bulk and ALDH positive CSC populations.

Based upon the favorable flow cytometry cell results with 9cUAB130, further tumorsphere studies were performed comparing carboplatin ± 9cUAB130. Untreated A2780 spheres demonstrated 16.0% ALDH positivity, and treatment with single agent 9cUAB130