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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.

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Conflict of interest statement

The authors have no financial conflicts of interest to declare.

Keywords

Retinoid; Ovarian cancer; Stem cell

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 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 $\times$ 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% CO₂ 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 (Stem-Cell 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 × 10⁶ 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 μ L 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 μ M on day 1. Carboplatin was added at a dose of 50 μ M (IC_{80}) on day 2, then media was replaced and cells were incubated for an additional 24 h. Cells were harvested and 200 μ L 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 μ M for 9cUAB30, 10 μ M for 9cUAB124, and >10 μ M for 9cUAB130 (Fig. 1A). A2780 cells treated with carboplatin yielded an IC_{50} of 15 μ M (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 $\pm$ 9cUAB130. Untreated A2780 spheres demonstrated 16.0% ALDH positivity, and treatment with single agent 9cUAB130 did not alter ALDH positivity (15.8%) (Table 2). Treatment with single agent carboplatin yielded an almost two fold increase in the population of ALDH positive cells (31.9%). Combination treatment with 9cUAB130 and carboplatin reduced ALDH positive tumorspheres back to

levels observed in untreated controls (14.2%), again suggesting that the combination of retinoid and carboplatin reduced ALDH positive marker expression, resulting in a potentially less aggressive phenotype (Fig. 3).

Tumorsphere viability

Manual counts of tumorspheres were performed in 6-well plates after treatment with carboplatin, 9cUAB130, or the combination. Treatment with 9cUAB130 or carboplatin reduced sphere viability to 58.4% and 40.3%, respectively. Combination treatment with 9cUAB130 and carboplatin further reduced sphere viability to 28.0% (Table 2).

Tumorspheres were also morphologically evaluated via light microscopy. Untreated spheres were well organized and measured greater than 100 μm (Fig. 3A). Addition of 9cUAB130 alone increased cellular debris, but did not appear to significantly decrease sphere size (Fig. 3B). Carboplatin alone at 50 μM led to further sphere disorganization. Spheres were noted to be reduced in size, but individual cells were larger and edematous (Fig. 3C). The combination of 9cUAB130 and carboplatin further disrupted sphere formation and decreased sphere size (Fig. 3D).

Tumor growth after ex vivo treatment with retinoids and chemotherapy

Mice injected with A2780 tumor cells and treated with 9cUAB130, carboplatin or the combination, were observed for tumor growth for 40 days after injection (Fig. 4). Four of four mice injected with untreated cells developed flank tumors, with an average tumor volume of 211 mm^3 . Three of four mice injected with cells treated with 9cUAB130 developed tumors, with an average volume of 42 mm^3 . Three of four animals injected with A2780 cells treated with carboplatin developed flank tumors, with an average volume of 18 mm^3 . None of the mice injected with cells treated with the combination of 9cUAB130 and carboplatin developed tumors within the 40 day observation period.

Discussion

Several *in vitro* and *in vivo* studies have evaluated the ability of retinoids to enhance response to traditional chemotherapy. Caliaro et al. showed in ovarian cancer cell lines that are sensitive to ATRA, pretreatment with ATRA followed by cisplatin enhances cytotoxicity compared to cisplatin alone [8]. Aebi et al. found that combination treatment also enhanced apoptosis in ovarian cancer cell lines [7]. A similar study by Supino et al. demonstrated the ability of a synthetic retinoid, fenretinimide (4HPR), to increase cytotoxicity compared to cisplatin alone [19]. Retinoids have also been shown to enhance response to traditional chemotherapy in animal models. Formelli et al. found in IGROV-1 xenografts that intraperitoneal administration of 4HPR significantly increased the survival time of treated animals, and enhanced the antitumor activity of cisplatin [6]. Suzuki et al. demonstrated that another synthetic retinoid, TAC-101 (4-[3,5-bis (trimethylsilyl) benzamido] benzoic acid) could improve tumor inhibition over traditional chemotherapy in a mouse ovarian cancer xenograft model [20].

Despite such promising preclinical studies, results from phase I/II clinical trials have been modest at best. In a study of 16 patients with recurrent ovarian cancer, Rustin et al. found

that etretinate showed very little activity as 12 patients progressed on treatment [21]. Columbo et al. evaluated preoperative administration of 4HPR in patients with newly diagnosed ovarian cancer and found no significant response [22]. While preclinical studies support the role for retinoids in treatment of ovarian cancer, clinical results indicate a need for more effective, less toxic retinoids to augment traditional treatment.

In our study, we evaluated the efficacy of three novel retinoid compounds in combination with single agent carboplatin-based chemotherapy. 9cUAB30, which has been approved by the National Cancer Institute for a breast cancer chemoprevention trial, is a retinoid that is effective, but toxic especially with regard to hyperlipidemia. 9cUAB124 and 9cUAB130 are part of a newer class of retinoids, thought to have similar efficacy with an improved side-effect profile. We evaluated the ability of these retinoids to augment the response to chemotherapy.

Our findings indicate that in the A2780 cell line combination treatment with retinoid and carboplatin was more effective than either single-agent alone. Notably, the IC₅₀ of 9cUAB30 was 1 μ M; in previous studies of 9cUAB30 in leukemia cell lines, the standard dose employed was 5 μ M [15], indicating that this ovarian cancer cell line was quite sensitive to this novel retinoid. Additionally, 9cUAB124 and 9cUAB130 increased cytotoxicity compared to chemotherapy alone, supporting the use of this cell line in animal studies.

Significant research has been performed evaluating the sensitivity of the CSC population to treatment with a variety of agents. It is thought that CSCs are responsible not only for recurrence of malignancy, but also for its associated chemo resistance [17]. ALDH has been associated with CSCs in solid tumors mainly by its coexpression in cells that also express other stem cell markers [23–25]. ALDH is also known to interact with the retinoic acid pathway by inducing tissue-specific oxidation of retinal to retinoic acid in several tissue types [26], including ovary [27]. The ALDEFLUOR assay has been used in ovarian tissue samples to demonstrate the upregulation of ALDH positive cells [27, 28]. Interestingly, inhibiting ALDH activity using nanoliposomal siRNA sensitized the cells to chemotherapy [29]. In our study, we found that untreated A2780 cells had a low percentage of ALDH positive cells, and although there was no significant reduction in CSC marker expression following treatment with retinoids, it is possible that both the non-CSCs and CSCs were equally affected by the retinoid therapy. Treatment with carboplatin produced cytotoxicity, the ALDH positive population was not depleted, and the population that survived treatment was enriched for a CSC phenotype, similar to results found by Rizzo et al. in a study of stem cell-like side populations [18]. Combination treatment with 9cUAB130 and carboplatin decreased the stem cell population compared to carboplatin alone, indicating that this combination treatment may be more favorable compared to traditional chemotherapy. This combination effect could be a result of the retinoid sensitizing the cells to carboplatin by inhibiting ALDH activity, however further studies are needed to elucidate the mechanism of inhibition.

While 9cUAB30 and 9cUAB130 yielded positive results in cytotoxicity studies, 9cUAB130 resulted in a favorable profile in ALDH activity analyses, and was therefore selected for use

in tumorsphere studies. The tumorsphere results were consistent with data from the flow cytometry cell analyses. This further suggests that combination treatment with 9cUAB130 and carboplatin enhanced cytotoxicity while reducing CSC marker expression and was more effective compared to treatment with carboplatin alone. These data are also supported by results from *ex vivo* treatment and tumorigenicity studies.

Collectively, our results indicate that the combination of 9cUAB130 and carboplatin is not only cytotoxic to A2780 cells, but also has potential to decrease the population of CSCs compared to treatment with carboplatin alone. These results are supported by decreased tumor burden (both number and volume) in animals injected with cells treated with combination therapy compared to animals treated with single agent carboplatin. These data imply that combination treatment with this novel retinoid and carboplatin could have implications for decreased chemoresistance in a clinical setting. Further translational and clinical studies are warranted to evaluate this treatment regimen.

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Fig. 1.

A2780 cell viability after treatment with retinoid or carboplatin. Cells were plated at 1000 cells per well for 24 h. Retinoids were added on days 1, 3, and 5 after plating for dose response assessment. On day 7, cell viability was determined by measuring cellular ATP levels using an ATPLite luminescence-based assay, and 9cUAB30 resulted in the most dramatic cytotoxicity (A). For carboplatin dose response, carboplatin alone was added on day 3 and ATPLite assay was performed on day 7, yielding an IC_{50} of 15 μM (B). All assays

were performed with six replicates in two separate experiments and results are reported as the mean and standard error.

Fig. 2.

A2780 cell viability after treatment with carboplatin and 9cUAB30, 9cUAB124, and 9cUAB130. Cells were plated 1000 cells per well for 24 h. In combination treatments, retinoid was added on days 1, 3, and 5; carboplatin was added on day 3. ATPlite assay was performed on day 7. Combination treatment of A2780 cells with each retinoid and carboplatin increased cytotoxicity over retinoid or carboplatin alone. All assays were performed with six replicates in two separate experiments, and results are reported as the mean and standard error.

Fig. 3.

A2780 spheres after treatment with 9cUAB130, carboplatin, or combination. Effect of 9cUAB130 and carboplatin treatment on primary tumorsphere formation from A2780 ovarian cancer cell line. Cells were plated at 100,000 cells/well in ultra-low attachment plates in serum-free MEBM-2. Spheres were treated with vehicle control (A), 9cUAB130 (B), carboplatin (C), and combination carboplatin and 9cUAB130 (D). Spheres ($> 50 \mu\text{m}$) were counted after completion of treatment. Scale bar represents $100 \mu\text{m}$.

Fig. 4.

Tumor growth after *ex vivo* treatment of A2780 cells with retinoids and chemotherapy. A2780 cells were plated at 200,000 cells/well for 24 h. Retinoid (at a dose of 10 μ M) was added on day 1, carboplatin was added (50 μ M) on day 2, and media was replaced on day 3. On day 4, equal volumes of cells were injected with matrigel into the flank of athymic nude mice. Tumor size was determined by the product of the 2 greatest diameters. Results are reported as mean and standard error.

Table 1

ALDH activity in A2780 cells.

Treatment ^a	Cell viability (% control)	% total with ALDH1 activity
Untreated	100.0 (±0.0)	3.3 (±0.1)
9cUAB30	94.5 (±12.2)	4.9 (±0.3)
9cUAB130	96.6 (±1.8)	2.3 (±0.7)
Carboplatin	21.4 (±5.3)	27.3 (±5.4)
9cUAB30 + carboplatin	22.1 (±2.9)	25.7 (±2.2)
9cUAB130 + carboplatin	18.1 (±5.0)	6.7 (±0.9)

^a A2780 cells were plated at 200,000 cells/well in 6-well attachment plates in standard media for 24 h. Retinoid (at a dose of 10 μ M) was added on day 1, carboplatin was added at its IC₈₀ (50 μ M) on day 2, and media was replaced on day 3. Cells were processed for flow cytometry on day 4. Results are pooled from 3 separate experiments and reported as mean and standard error.

Table 2

ALDH activity in A2780 spheres.

Treatment ^a	Sphere viability (% control)	% total with ALDH1 activity
Untreated	100.0 (±7.5)	16.0 (±5.0)
9cUAB130	58.4 (±10.3)	15.8 (±7.4)
Carboplatin	40.3 (±7.9)	31.9 (±5.2)
9cUAB130 + carboplatin	28.0 (±4.1)	14.2(±4.1)

^a A2780 spheres were plated in 6-well plates for 24 h. 9cUAB130 (at a dose of 10 μ M) was added on day 1, carboplatin was added (50 μ M) on day 2, and cells were processed for flow cytometry on day 4. Results are pooled from three separate experiments and reported as mean and standard error.