The Small Heat Shock Protein αB-Crystallin is Critical to Cell Survival in Response to the Phytochemical Withaferin A in Cisplatin-Resistant Ovarian Cancer Cells

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The Small Heat Shock Protein αB-Crystallin is Critical to Cell Survival in Response to the Phytochemical Withaferin A in Cisplatin-Resistant Ovarian Cancer Cells

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Submitted in Partial Completion of the Requirements for Departmental Honors in Biological Sciences

Bridgewater State University

May 12, 2020

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1. Abstract

Withaferin A (WA), the active-phytochemical of the medicinal plant Withania somnifera, promotes apoptosis in cancer cells. Although its mechanism remains unclear, WA has been shown to upregulate heat shock proteins (Hsps) which protect against diverse cellular insults. The small Hsps, αB-Crystallin and Hsp27, confer resistance to chemotherapy in several cancer cell lines, however, the role of WA on αB-Crystallin and Hsp27 expression in drug resistance remains unknown. Since the majority of ovarian tumors eventually recur in a drug-resistant form leaving patients few treatment options, the goal of this study is to explore the molecular mechanism induced by WA in a cisplatin-resistant ovarian cancer cell line (OVCAR8R) as compared to its cisplatin-sensitive syngeneic counterpart (OVCAR8). Importantly, I found that αB-Crystallin and Hsp27 are constitutively expressed in the OVCAR8R cells, while OVCAR8 cells do not endogenously express these Hsps, suggesting that overexpression of Hsps may confer resistance to chemotherapy and promote a more aggressive tumor type. To determine the effect of WA, OVCAR8 and OVCAR8R cells were treated with 0.5, 1.0, 2.5, 5.0 or 7.5µM WA. Morphological studies show WA-induced cytotoxicity in both cell lines, however the OVCAR8R cells remain viable at 5.0 and 7.5µM WA while few OVCAR8 cells remain viable at these doses. Flow cytometry supports this as the percent of Annexin positive cells following WA treatment increases in a dose-dependent manner in OVCAR8 cells, while the percent of Annexin positive OVCAR8R cells is negligible compared to controls at high doses of WA. Following WA treatment for 24h, the endogenous level of αB-Crystallin is upregulated in a dose dependent manner in the cisplatin-resistant OVCAR8R cells, while the cisplatin-sensitive OVCAR8 cells upregulate αB-Crystallin and Hsp-27 at 2.5 and 5.0µM WA. Experiments are underway to systematically determine the role of αB-crystallin in conferring resistance of ovarian cancer cells to apoptosis. αB-Crystallin will be silenced by CRISPR-Cas9 in the OVCAR8R and clones will be selected to address the role of αB-Crystallin on WA-induced
apoptosis. Conversely, wildtype αB-Crystallin and αB-Crystallin pseudophosphorylation mutants that alter its chaperone activity will be overexpressed in the OVCAR8 cells to determine the role of this protein in conferring resistance to WA-induced apoptosis. Together, this research aims to elucidate the effectiveness of WA as a potential therapy for ovarian cancer cells that acquire resistance to platinum-based therapies.
2. Introduction

2.1 Platinum-Resistant Ovarian Cancer

Ovarian cancer is the fifth leading cause of cancer-related deaths among women in the United States [1,3]. Amongst solid gynecological tumors, ovarian cancer is the deadliest gynecological malignancy [2,3]. The majority of patients present with advanced disease, for which the standard treatment is surgery and platinum-based chemotherapy [5,2]. Despite initial response to treatment, the majority of ovarian cancer patients develop recurrent disease within three years [2,3]. While initial recurrence is frequently cisplatin-sensitive, patients eventually develop resistance to cisplatin treatment [4]. Cancer cell resistance to platinum-based chemotherapies may be intrinsic, present before treatment, or acquired, induced by therapy. Resistance mechanisms, whether intrinsic or acquired, pose a major clinical problem in the treatment of ovarian cancer and are the main contributing factor in cancer-associated mortality, as such, a better understanding of acquired chemoresistance is clinically important [4]. Numerous mechanisms of platinum-resistance have been identified including enhanced DNA repair, disruption of apoptosis, reduced platinum accumulation, and the intracellular inactivation of cisplatin [17]. Resistant cells acquire one or more mechanisms contributing to the multifactorial nature of cisplatin resistance [18]. A prominent tool to gain insight to resistance mechanisms is the selection of drug-resistant lines derived from increasing drug concentrations [17]. Chowanadisai and Messerli et al. derived an ovarian cancer cisplatin-resistant cell line (OVCAR8R) through serial exposure of OVCAR8 cells to sub-lethal concentrations of cisplatin [17]. In their model, resistance was determined to be caused by cisplatin sequestration, increased expression of anti-apoptotic pathways, cytokines, and an increased mesenchymal expression profile [17]. Therefore, utilizing this in vitro model, further understanding of the mechanisms that underlie resistance to platinum-based therapies can be
determined and additional therapeutics to target these cells that are refractory to first-line treatments can be identified.

2.2 Platinum-Based Chemotherapeutics

Platinum-based drugs, in particular, cisplatin (cis-dichlorodiammineplatinum (II)) is highly effective in the treatment of ovarian cancer [6]. Cisplatin is a square planar inorganic complex that can cross the cell membrane, pass through the cytosol, and into the nucleus to interact with genomic DNA (Figure 1). Cisplatin is activated by hydrolysis, wherein chloride ions are substituted with water molecules, resulting in a strong electrophile [7]. The main biochemical mechanism of action of cisplatin involves the binding of the drug to DNA within the nucleus [8]. Due to their high nucleophilicity and accessibility, the N7 atoms of guanine and adenine in the major groove are the most reactive sites on DNA for platinum coordination [6]. The reaction of cisplatin with DNA may lead to the formation of numerous structurally diverse adducts (Figure 1B) [7]. The initial binding of cisplatin to DNA forms monofunctional DNA adducts [6]. A monofunctional

![Figure 1: (A) Structure of cis-dichlorodiammineplatinum (II) adapted from author, MC, 1997. (B) Cisplatin DNA Adducts: (a) Cisplatin bound monofunctionally to guanine (X—original chloride, or a hydroxyl group); (b) inter-strand crosslink; (c) cisplatin guanine—protein crosslink; (d) GpG-intrastrand crosslink; (e) GpNpG intrastrand crosslink (N represents a base); (f) ApG-intrastrand crosslink. Adopted and modified according to Crul et al. 2002.](image)
DNA adduct is a single strand of DNA covalently attached to a chemical [19]. However, most react further to produce inter- and intra-strand crosslinks (Figure 1B) [6]. This damage induced upon cisplatin binding to genomic DNA may inhibit transcription and/or DNA replication [8]. These alterations in DNA processing would initiate the cytotoxic processes of cisplatin ultimately leading to cancer cell death [6]. Inhibition of DNA replication prevents cancer cells from continuously dividing and underlies the effectiveness of cisplatin in cancer treatment.

2.3 Natural Products ~ Withaferin A

Plant constituents, known as phytochemicals, have gained the status of clinically-used modern medicines and have been used for treating various diseases including malignant tumors [7]. Phytochemicals are secondary metabolites that are biologically active and can interact directly or indirectly with various cellular components [9]. Numerous phytochemicals are under investigation that may be employed as secondary treatments to target cisplatin-resistant cells in various malignancies. The use of phytochemicals in the treatment of ovarian cancer holds promise as they may synergize with or target resistant cells with potentially fewer adverse side effects than platinum-

Figure 2: (A) *Withania somnifera*, an important component of Ayurvedic medicine. Adapted from author, BH, 2020. (B) Structure of Withaferin A adapted from author, MR, 2016. Withaferin A is a steroidal lactone derived from the medicinal plant *Withania somnifera*.
based chemotherapies [9,7]. *Withania somnifera* is a well-known Ayurvedic medicinal plant originating from the dry regions of Asian countries such as India (Figure 2A) [10]. Ayurvedic medicine is a type of holistic medicine, also known as complementary and alternative medicine, that employs plant derivatives to treat diverse pathologies [21]. It has broad therapeutic applications, including anti-inflammatory activities, action on the immune system, and has been shown to have an emerging effect on cancer inhibition [10]. The abundant therapeutic applications of *W. somnifera* are related to the presence of alkaloids and lactones found at different levels within the plant parts (roots, leaves, stems). The anticancer properties of *W. somnifera* are attributable to withanolides, a class of bioactive constituents isolated from the plant parts [9]. Several studies have reported a rich source of withanolides from the leaves of *W. somnifera* found to be cytotoxic to cancer cells [10].

Withaferin A, the principal active-phytochemical of *W. somnifera*, was the first anti-tumor withanolide isolated from the leaves of the plant in 1967 (Figure 2B) [9]. Withaferin A has been suggested as a potential anti-cancer compound shown to prevent tumor growth, angiogenesis, and metastasis [11]. Biochemical processes that underlie the anticancer effects of Withaferin A include the reinforcement of cellular antioxidant and/or detoxification system; suppression of inflammatory pathways; selective inhibition of tumor cell proliferation and induction of apoptosis; suppression of tumor angiogenesis; blockade of epithelial-to-mesenchymal transition (EMT), tumor invasion, and metastasis; alteration of tumor cell metabolism; immunomodulation; and eradication of cancer stem cells [10,11]. Withaferin A has been used alone and in combination with cisplatin to treat cisplatin-sensitive and -resistant ovarian cancer. Kakar *et al.* demonstrated that the combination of Withaferin A and cisplatin reduces the dosage requirement of cisplatin to achieve therapeutic effects, therefore, having a significant potential to serve as a treatment for
cisplatin-resistant ovarian cancers [11]. Studies on breast cancer models have demonstrated that Withaferin A treatment inhibits experimental EMT and the inhibition can be contributed to its effects on vimentin [24,25]. Vimentin is an intermediate filament essential for cell motility and migration [24]. Overexpression of vimentin correlates with metastatic disease, EMT induction, and poor prognosis [24]. Several studies have demonstrated Withaferin A treatment suppresses expression of vimentin and initiates its disassembly [24,25,26]. Loss of vimentin expression nearly eliminates cell migration demonstrating the therapeutic potential of Withaferin A as an anti-metastatic agent [24,25].

2.4 Apoptosis & Heat Shock Proteins

Apoptosis is a form of programmed cell death, a molecular mechanism, by which cells are eliminated [1,13,15]. It plays a critical role as a homeostatic mechanism, maintaining cell populations, during development and aging [13,15]. Apoptosis also occurs as a defense mechanism when cells are damaged by disease [15]. Apoptosis is marked by its morphological characteristics,
including cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation resulting in apoptotic bodies (Figure 3A) [12]. Following fragmentation of the cell, apoptotic bodies are engulfed by nearby macrophages, avoiding inflammation [12,14]. Macrophage uptake of apoptotic cells is signaled for by the externalization of the phospholipid phosphatidylserine on the cell membrane [15]. Phosphatidylserine is located within the inner leaflet of the plasma membrane in healthy cells and the translocation from the inner leaflet to the outer leaflet of the plasma membrane facilitates macrophage recognition allowing for the uptake and disposal of the apoptotic bodies (Figure 3B) [15].

Apoptosis is a tightly regulated molecular pathway activated by both intracellular and extracellular signals [13]. It can be induced through two pathways: the intrinsic or mitochondrial pathway, and

Figure 4: Schematic Overview of Apoptotic Pathways. Apoptosis can occur by the intrinsic (mitochondrial) pathway, or the extrinsic (death receptor) pathway. The intrinsic pathway relies on the release of cytochrome c from the mitochondria, which binds to APAF-1 and procaspase-9, resulting in oligomerization of the “apoptosome” and activation of caspase-9. The extrinsic pathway depends upon the binding of a death ligand to its receptor, triggering the recruitment of adaptor proteins (FADD) and procaspase-8 resulting in the activation of caspase-8. Both pathways converge upon the activation of caspase-3, which cleaves cellular substrates that lead to the demise of the cell. Image generated on BioRender.
the extrinsic or death receptor pathway (Figure 4) [1,13,15]. The intrinsic pathway is activated by the presence of an insult to the cell such as chemotherapy or radiation initiating downstream effects leading to mitochondrial membrane permeabilization [1,13]. The permeabilization of the mitochondrial membrane allows for the release of the pro-apoptotic protein, cytochrome c, into the cytosol [1,13,15]. Following the release of cytochrome c, the apoptosome is formed, through protein-protein interactions with cytochrome c, apoptotic protease-activating-factor-1 (APAF-1), and procaspase-9 [13,15]. The formation of the apoptosome activates caspase-9, in turn, activating the executioner caspase, caspase-3, leading to the disassembly of the cell [13,15].

The extrinsic pathway is activated by extracellular signals, known as death ligands, that bind to the tumor necrosis factor (TNF) family death receptors (Figure 4) [1,13,15]. Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) bind to Fas or TRAIL receptors, respectively, triggering the recruitment of adaptor proteins known as Fas-associated death domain proteins (FADD) [1,13,15]. Initiator procaspase-8 is recruited, binds FADD, activating caspase-8, in turn, cleaving procaspase-3 into active caspase-3 initiating the disassembly of the cell [1,13,15]. The extrinsic and intrinsic pathways converge on the activation of the executioner caspase, caspase-3, which cleaves various substrates resulting in the morphological and biochemical changes seen within cells undergoing apoptosis [1,13,15].

Disruption of apoptosis can occur through a variety of mechanisms causing tumor initiation, progression, and treatment resistance [14]. Recent research has shown heat shock proteins (HSPs) bind and inhibit several molecules in the apoptotic pathway (Figure 5) [2]. HSPs are stress-inducible molecules known for their molecular chaperone activities including protein folding, cellular protein trafficking, and anti-aggregation of proteins [2]. Small HSPs (sHSPs), chaperones
with the molecular weight of 12-43 kDa, are widely implicated in biological processes including apoptosis, in addition to their chaperone function [2]. sHSPs, HSP27, and αB-crystallin, have been shown to have anti-apoptotic properties in cancer cells (Figure 5) [2]. HSP27 has been shown to interact with cytochrome c in the cytoplasm inhibiting the formation of the apoptosome, therefore, inhibiting apoptosis [2]. αB-crystallin has been shown to interact with pro-caspase-3 inhibiting the activation of caspase-3, in turn, inhibiting apoptosis [16].
3. Materials & Methods

3.1 Mammalian Cell Culture

The ovarian cancer cell lines, OVCAR8 (Cisplatin Sensitive) and OVCAR8R (Cisplatin Resistant) were provided by our collaborator (Shanta Messerli) and were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. Cells were maintained and incubated at 37°C in a 5% CO₂ environment. OVCAR8R cells were maintained in 5µM Carboplatin (Sigma).

3.2 Withaferin A Treatment

Withaferin A (EMD Millipore) was diluted in DMSO according to the manufacturer’s instructions. Cells were plated 24 hours prior to treatment at a concentration of 250,000 cells/mL. Cells were treated with concentrations of 0, 0.5, 1.0, 2.5, 5.0 and 7.5µM Withaferin A, then allowed to incubate for 24 hours.

3.3 Annexin Staining and Flow Cytometry

Cells were treated as described, then collected and washed in 1 mL of 1x Binding Buffer at 300 x g for 10 minutes. The cell pellet was resuspended in 100 µL of 1x Binding Buffer. 10µL of Annexin V-FITC was added prior to a 15-minute incubation in reduced lighting. Cells were washed in 1 mL of 1x Binding Buffer at 300 x g for 10 minutes. The cell pellet was resuspended in 500 µL of 1x Binding Buffer. 5 µL of Propidium Iodide (PI) solution was added immediately prior to analysis by flow cytometry.
3.4 Cell Harvest and Western Blot Analysis

Cells were treated as described, and both adherent and non-adherent cells were collected for protein analysis. Cells were scraped using a cell scraper after which cells were centrifuged, washed in PBS with Protease Inhibitors and re-suspended in an equal volume of PBS and 2x Laemmli buffer. DNA was sheared using a 26-gauge needle and 10 μL lysates were loaded into each well of a 15% polyacrylamide gel. SDS-PAGE was performed on whole-cell lysates and proteins were transferred to a PVDF membrane. The membrane was blocked in 10 mL (5% milk in Tris-Buffered Saline Tween-20, TBST) for 1 hour. The membrane was washed 4 times in TBST for 5 minutes and blocked in 5% Milk-TBST. Membranes were incubated for 1 hour each in primary antibodies diluted in 1% milk in TBST. Primary antibodies included the following: 1:1000 dilution of Hsp-27 (SPA-800), Hsp-70 (SPA-822), or alpha-B Crystallin (SPA-222) (Enzo); 1:2500 dilution of anti-Tubulin (Sigma, T5168), 1:1000 dilutions of Caspase-3 (#9665 Cell Signaling), 1:1000 PARP-1 (#9542 Cell Signaling), primary antibody dilutions. The membrane was washed 4 times in TBST for 5 minutes and incubated at room temperature for 1 hour on a rocker in 10 mL (1% milk in TBST) 1:10,000 secondary HRP-conjugated anti-rabbit antibody dilution (SC-2004 Santa Cruz Biotechnology). The membrane was washed 4 times with TBST for 5 minutes, then once in dH₂O for 5 minutes, followed by incubation in 10 mL ECL Chemiluminescence for 2 minutes and exposed to film.

3.5 Wound Healing Assay

The ovarian cancer cell lines, OVCAR8 (Cisplatin Sensitive) and OVCAR8R (Cisplatin Resistant) Cells were plated and allowed to reach 100% confluence. A P10 pipette tip was used to scrape a straight line within the cell monolayer. Media was aspirated, cells washed with 500μL DMEM,
and then replaced with 1 mL DMEM. Reference markings were created using a razor blade on the outer bottom of the plate close to the scratch. An initial image was acquired then returned to a tissue culture incubator, 37°C in a 5% CO₂ environment. Plates were removed from the incubator periodically, imaged, then returned to continue incubation.

3.6 Puromycin Death Curve

OVCAR8R cells were plated in 24 well plates at a density of 40,000 cells/well. Twenty-four hours after plating, cells were treated with increasing concentration of puromycin (Santa Cruz) with doses ranging from 0-10.0 μg/mL. Detached cells and media were removed every three days and the same concentration puromycin were added to each well to establish the lowest dose at which no cells remained viable to allow selection of cells that express a puromycin-resistance gene (See Figure 14A for details).

3.8 Transfection of CRISPR Plasmids and Selection of Clones

OVCAR8R cells were transfected with 1 μg of CRISPR alpha-B Crystallin-GFP vector, 1 μg of CRISPR alpha-B Crystallin HDR-RFP vector or 1 μg of control vector (pEGFP-N1, CLONTECH) and allowed to recover for 48 h (Figure 13). Cells were then transferred to 100mm plates and clones stably expressing these constructs were selected by growth in puromycin (Life Technologies) for 3 weeks. Individual colonies were selected and rescued using sterile cloning cylinders (Figure 14B). Clones were trypsinized from each cloning cylinder and puromycin-resistant clones were propagated and examined for expression of αB-crystallin by immunoblotting with a monoclonal antibody for alpha-B Crystallin (Enzo) as described above.
4. Results

4.1 Morphological and Protein Analysis of OVCAR8 and OVCAR8R cell lines

In order to characterize the morphological and molecular traits involved in the resistance of cells to cisplatin, untreated OVCAR8, and OVCAR8R cells were analyzed for morphology and expression of HSPs, full-length Caspase-3, and Vimentin. (Figure 6). Cisplatin-Resistant OVCAR8R cells appear elongated, and spread out on the surface of the flask, whereas the Cisplatin-Sensitive OVCAR8 cells appear smaller and are more spindle-shaped (Figure 6A). Expression of HSP27 and αB-Crystallin was not detected in the OVCAR8 cell line, however, both HSP27 and αB-Crystallin are constitutively expressed in OVCAR8R (Figure 6B). HSP overexpression correlates with expression of vimentin, a well-characterized marker that supports a more metastatic phenotype of the chemoresistant cells (OVCAR8R) (Figure 6B). Furthermore, the resistant cells express lower levels of full-length caspase-3 which supports they may be less likely to enter into an apoptotic program as compared to their cisplatin-sensitive counterpart (Figure 6B).

![Figure 6: Morphological and Protein Analysis of OVCAR8 and OVCAR8R Cell Lines.](image)

Figure 6: Morphological and Protein Analysis of OVCAR8 and OVCAR8R Cell Lines. (A) Morphological comparison of OVCAR8 (panel A) and OVCAR8R cells (panel B) (B) Western Blot analysis on lysates from OVCAR8 and OVCAR8R cells. Untreated OVCAR8 and OVCAR8R cells were analyzed for expression of HSPs, a key mediator of apoptosis (Caspase-3), and a marker of metastatic potential (Vimentin). Tubulin expression shows that equal protein is loaded in each well.
4.2 Morphological and Flow Cytometric Analysis of WA-treated OVCAR8R cells

To determine the effect of WA on cell survival, cells were incubated with 0.5µM, 1.0µM, 2.5µM, 5.0µM, or 7.5µM WA for 24hrs. Cellular morphology shows WA-induced cytotoxicity in both OVCAR8 and OVCAR8R cells (Figure 7 and 8). While WA is cytotoxic to both cell lines, the OVCAR8R cells remain partially viable and are still adhered to the plate at 7.5µM WA while no OVCAR8 cells appear viable and none retain attachment to the plate at this dose (Figure 7 and 8). Flow cytometry supports this finding as the percent of Annexin positive cells following WA treatment increases in a dose-dependent manner in OVCAR8 cells, while the percent of Annexin positive OVCAR8R cells is negligible compared to controls at high doses of WA (Figure 9, Panel A). These results underscore that the cells that have acquired resistance to cisplatin have also acquired more general chemoresistance given their decreased sensitivity to WA-induced apoptosis.

Figure 7: Morphological Analysis of WA-Treated OVCAR8 Cells. Cells were incubated in DMEM with A) vehicle (DMSO), B) 0.5µM WA, C) 1.0µM WA, D) 2.5µM WA, E) 5.0µM WA, or F) 7.5µM WA. Cells with increasing concentrations of WA progressively detach from the plate and exhibit increased nuclear condensation and cell shrinkage.
Figure 8: Morphological Analysis of WA-Treated OVCAR8R Cells. Cells were incubated in DMEM with A) vehicle (DMSO), B) 0.5μM WA, C) 1.0μM WA, D) 2.5μM WA, E) 5.0μM WA, or F) 7.5μM WA. Cells with increasing concentrations of WA progressively detach from the plate and exhibit increased nuclear condensation and cell shrinkage.

Figure 9: Flow Cytometric Analysis of WA-Treated OVCAR8 and OVCAR8R Cells. (A) Treatment with 5.0 and 7.5μM WA induces an increase in the percent Annexin positive cells in the Cisplatin Sensitive OVCAR8 cells but not in the Cisplatin Resistant cells. (B) A representative of one experiment is shown in which Cisplatin Sensitive (upper panels) or Cisplatin Resistant (lower panels) cells were treated with vehicle (DMSO) in the control sample, or 5.0μM WA, and collected 24 hours later. Cells were stained with Annexin V-FITC and PI. Viable cells are negative for both Annexin V-FITC and PI (Lower Left Quadrant). Apoptotic cells stain positively for Annexin V-FITC (Lower and Upper Right Quadrants), which binds to phosphatidylserine, while necrotic cells stain positively for PI (Upper Left Quadrant).
4.3 Western Blot Analysis on lysates from WA-treated OVCAR8 and OVCAR8R cells.

Following WA treatment, cells were collected for analysis of apoptosis and protein expression, specifically for the small HSPs, αB-Crystallin, and HSP27 along with two proteins that indicate apoptosis, Caspase-3, and its substrate poly (ADP-ribose) polymerase, or PARP (Figure 10, 11, 12). Full-length Caspase-3 and PARP appear more abundant in the OVCAR8 cells as compared to the OVCAR8R cells, however, both cell lines show cleavage of full-length Caspase-3 (as evidenced by decreasing expression with increased dose of WA) and PARP at high doses of WA (Figure 10).

![Western Blot Analysis](image)

Figure 10: **Western Blot Analysis on Lysates from WA-Treated OVCAR8 and OVCAR8R Cells.** Western blot analysis of key mediators of apoptosis indicates WA induces PARP and Caspase-3 activation, in a dose dependent manner. PARP and Caspase-3 are activated (as evidenced by expression of the cleaved form of these proteins) when cells are treated with WA. Tubulin expression shows that equal protein is loaded in each well.

The finding that the cisplatin-resistant cells express lower levels of apoptotic proteins support that OVCAR8R cells may be less likely to enter into an apoptotic program. OVCAR8R cells were found to upregulate the endogenous level of αB-Crystallin in a dose-dependent manner, while OVCAR8 cells upregulate αB-Crystallin and HSP27 at 5.0µM WA coincident with downregulation of Vimentin (Figure 11 and 12). The upregulation of HSPs following treatment with WA reveals that cells are simultaneously upregulating a cellular stress response to WA yet may be less likely to metastasize. The significance of this finding is an area of further exploration.
As experiments using the knockout clones are still underway, all Western blot data will be subjected to densitometry in order to determine protein levels as compared to tubulin. This data will allow for a more quantitative measure of the increased expression of HSPs as well as the WA-induced downregulation of vimentin and decreased level of caspase-3.

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**Figure 11:** Western Blot Analysis for HSP Expression and Metastatic Markers on Lysates from WA-Treated OVCAR8R Cells. (A/B) Western blot analysis of OVCAR8R cells reveals αB-Crystallin is upregulated in WA-Treated OVCAR8R cells. (B) Western blot analysis of OVCAR8R cells indicates the constitutive expression of HSP27 and Vimentin. Recombinant αB-Crystallin was used as a control. Tubulin expression shows that equal protein is loaded in each well.

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<tr>
<th>OVCAR8R</th>
<th>WA (µM)</th>
<th>WB</th>
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<tbody>
<tr>
<td>αB-Crystallin</td>
<td>C 0.5 1.0 2.5 5.0 7.5</td>
<td>αB-Crystallin</td>
</tr>
<tr>
<td>Hsp27</td>
<td></td>
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<tr>
<td>Vimentin</td>
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<td>Tubulin</td>
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**Figure 12:** Western Blot Analysis for HSP Expression and Metastatic Markers on Lysates from WA-Treated OVCAR8 Cells. Western blot analysis of OVCAR8 cells reveals αB-Crystallin and HSP27 are upregulated at the 5.0µM dose of WA coincident with downregulation of Vimentin. Recombinant αB-Crystallin was used as a control. Tubulin expression shows that equal protein is loaded in each well.

<table>
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<tr>
<th>OVCAR8</th>
<th>WA (µM)</th>
<th>WB</th>
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<tbody>
<tr>
<td>αB-Crystallin</td>
<td>C 0.5 1.0 2.5 5.0 7.5</td>
<td>αB-Crystallin</td>
</tr>
<tr>
<td>HSP27</td>
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<td>Vimentin</td>
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<td>Tubulin</td>
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4.4 Construction of Alpha-B Crystallin Knock-Out Clones.

These findings led to the question of whether αB-Crystallin plays a role in conferring resistance to apoptosis. To address this question, αB-Crystallin was silenced through the use of a CRISPR-Cas9 expression construct in OVCAR8R cells (Figure 13). The first steps in this experimental process were to perform a puromycin death curve wherein cells were treated with 0-10μg/mL puromycin.
in order to determine the lowest concentration that kills 100% of cells within five days from the start of puromycin selection. Few OVCAR8R cells remained viable at 0.25µg/mL while all cells at the 0.5µg/mL dose and above were dead and had detached from the plate. Therefore, 0.5µg/mL was determined to be the optimal working concentration to use for selection of the puromycin-resistant cells for the OVCAR8R cell line. Cells were transfected, selected, and rescued from 150mm plates and expanded in T25 flasks (Figure 14B). Following expansion, clones were analyzed by Western blot analysis to determine success of silencing αB-Crystallin.

4.5 Morphological and Protein Analysis of Alpha-B Crystallin Knock-Out Clones

Western blot analysis confirmed 15 successful αB-Crystallin knock-out clones (Figure 15). Clones CraB1A and CraB7A were selected for the continuation of the study (Figure 16). CraB1A and CraB7A show morphological and molecular characteristics similar to OVCAR8 cells, losing the phenotype of the OVCAR8R cells (Figure 16/17). Morphological analysis of CraB1A and CraB7A reveals the clones return to the cisplatin-sensitive cell morphology and lose the morphology they had prior to selection which was that of the cisplatin-resistant cells (Figure 17).
This finding underscores that αB-Crystallin is likely to be an important player in the conversion of OVCAR8 cells as they gained resistance to cisplatin.

4.6 Western blot analysis of key mediators of apoptosis of CrαB1A and CrαB7A

Clones were incubated with 0.5µM, 1.0µM, 2.5µM, 5.0µM or 7.5µM WA for 24hrs. Following treatment, cells were collected for analysis of apoptosis and protein expression, specifically for two proteins that indicate apoptosis, Caspase-3, and its substrate PARP (Figure 18). The silencing of
αB-Crystallin resulted in increased Caspase-3 and PARP cleavage following WA treatment following WA treatment (Figure 18). WA induced cleavage of Caspase-3 and PARP in CrαB1A and CrαB7A most closely resembles the OVCAR8 cells (Figure 18). However, since this experiment has yet to be replicated, this finding is preliminary requiring further experiments to determine this potential difference. Ongoing work aims to overexpress αB-Crystallin in the cisplatin-sensitive OVCAR8 cells to address whether the over-expression of αB-Crystallin confers resistance to apoptotic inducing agents, such as WA.
5. Discussion

This study demonstrates that αB-Crystallin is an important player in the conversion of OVCAR8 cells as they gained resistance to cisplatin. Western blot analysis supports that OVCAR8R cells constitutively overexpress the sHSPs, αB-Crystallin, and HSP27, while OVCAR8 cells do not endogenously express these proteins.

OVCAR8 and OVCAR8R cells following treatment with Withaferin A for 24hrs were subjected to Annexin staining and morphological analysis providing support that OVCAR8 cells undergo apoptosis in a dose-dependent manner, however, OVCAR8R cells do not enter into apoptosis as readily given their chemoresistant characteristics. Having obtained data underscoring that the cells that have acquired resistance to cisplatin have also acquired more general chemoresistance, expression of the small HSPs, HSP27, and αB-Crystallin, along with two apoptotic markers, Caspase-3 and PARP, were analyzed by Western blot. Western blot analysis revealed Caspase-3 and PARP expression is more abundant in the OVCAR8 cell line as compared to the OVCAR8R cell line supporting that cells that have acquired resistance to cisplatin are less likely to enter into an apoptotic program. It was also found that OVCAR8R cells upregulate the endogenous level of αB-Crystallin in a dose-dependent manner in response to treatment with Withaferin A. The finding that both HSP27 and αB-Crystallin are upregulated in the cisplatin-sensitive OVCAR8 cells after treatment with WA supports that the cells that gain chemoresistance may be those that upregulate these HSPs, a question that can be further addressed through immunocytochemical and apoptosis analysis. These findings led to the question of whether αB-Crystallin plays a role in conferring resistance to apoptosis. Silencing of αB-Crystallin through the use of a CRISPR/Cas9 expression construct revealed that αB-Crystallin knock-out clones, CrαB1A and CrαB7A, lose the phenotype of the OVCAR8R cells. Morphological analysis revealed, CrαB1A and CrαB7A, lose the
morphism they had prior to selection, that of the cisplatin-resistant cells, and return to the cisplatin-sensitive cell morphology. Of note, the clones CraB3A and CraB12A that were found to retain expression of αB-Crystallin (silencing of this protein was not successful via CRISPR/Cas9, shown in Figure 15) did not have altered morphology as compared to the OVCAR8R cell line (data not shown). Together, the observed morphological changes of the OVCAR8R cells after an alteration of only αB-Crystallin warrants further investigation. CraB1A and CraB7A cells following treatment with Withaferin A for 24hrs were subjected to Western blot analysis for the two apoptotic markers, Caspase-3 and PARP. Western blot analysis indicated that the levels of endogenous PARP in the CraB1A and CraB7A clones more closely match that of the OVCAR8 cells. Furthermore, silencing of αB-Crystallin resulted in a similar profile of Caspase-3 and PARP cleavage following WA treatment, most closely resembling the OVCAR8 cells and less like the OVCAR8R cells.

Importantly, the analysis of gene expression findings of our colleagues is supported by this study. Of 3330 genes analyzed, the fold increase in expression of the small HSPs, αB-Crystallin, and

<table>
<thead>
<tr>
<th>EMT Gene</th>
<th>Fold Increase in Expression in OVCAR8R versus OVCAR8</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1</td>
<td>6.9</td>
</tr>
<tr>
<td>Matrix Metallopeptidase 1 (collagenase)</td>
<td>6.9</td>
</tr>
<tr>
<td>Fibronectin I</td>
<td>5.9</td>
</tr>
<tr>
<td><strong>Alpha B Crystallin</strong></td>
<td><strong>5.4</strong></td>
</tr>
<tr>
<td>Vimentin</td>
<td>4</td>
</tr>
<tr>
<td>Hsp27</td>
<td>2.4</td>
</tr>
</tbody>
</table>

**TABLE 1: Characterization of Cell Lines** A list of selected probesets that show differentially expressed genes in the OVCAR8 versus OVCAR8R cells (From Supporting Information, S2 Table, 17). Of 3330 genes analyzed, the fold increase in expression of the small HSPs, Alpha B Crystallin and Hsp27, and Vimentin support the protein expression profile in our study. Also highlighted are MMPI and Fibronectin, two additional markers of metastatic potential.
HSP27, and Vimentin, support the protein expression profile in our study. Also highlighted are MMP1 and Fibronectin, two additional markers of metastatic potential. MMP1, a member of the Matrix metalloproteinases (MMPs) family of proteases that degrade the extracellular matrix during physiological processes, including embryonic development and tissue remodeling, have been subsequently found to be upregulated in nearly every tumor type [27, 28, 29]. MMPs are able to proteolytically process substrates in the extracellular matrix, in turn, leading to tumor invasion and metastasis [27, 28, 29]. Fibronectin is a matrix glycoprotein that plays a role in cell growth, differentiation, and processes such as wound healing and blood coagulation [30]. Increased Fibronectin expression has been shown to mediate various metastatic-promoting mechanisms, including tumor growth and invasion [30, 31]. Together, the overexpression of genes that support a more aggressive and metastatic phenotype in the OVCAR8R cells warrants further study of αB-Crystallin, especially given that its expression level is comparable to that of fibronectin, and more abundant than vimentin, well-known metastatic markers. Future experiments to address this question will involve transient overexpression of αB-Crystallin in the cisplatin-sensitive OVCAR8 cells. Following transfection and transient overexpression of wild-type αB-Crystallin, we can determine by immunocytochemistry whether expression of this small HSP will prevent apoptosis in cells after treatment with apoptotic inducing agents such as Withaferin A. Furthermore, this line of experiments can also define whether chaperone function is necessary for conferring resistance as we plan to overexpress a mutant αB-Crystallin in which three serine residues are altered to glutamate to mimic phosphorylation, an event that prevents HSP oligomerization.

Ongoing studies include analyzing the migration of cisplatin-resistant cells, cisplatin-sensitive cells and the αB-Crystallin knock-out clones. Wound healing assays along with trans-well migration assays will determine whether the OVCAR8R cells are more invasive in vitro and the effect of αB-
Crystallin and Withaferin A treatment on this characteristic. Taken together this research will help to elucidate the role of αB-Crystallin as well as the effectiveness of WA as a potential therapy for ovarian cancer cells that acquire resistance to platinum-based therapies.
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7. References


