Determining the role of fatty acid synthase in the mechanism regulating chemotherapy resistance in recurrent ovarian carcinoma

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Biology in the undergraduate colleges of The Ohio State University

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May 2016

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Abstract:

Fatty acid synthase (FAS), a 272 kDa protein complex regulated via the sterol regulatory binding element protein (SREBP) pathway, is implicated in intracellular *de novo* synthesis of fatty acids and is overexpressed in a variety of human epithelial tumors, including ovarian cancer. FAS demonstrates a role in mediating cellular proliferation, survival, and migration and invasion, while decreasing the rate of apoptosis within the cells, especially when upregulated. The goal of the present study is to investigate the role of FAS-mediated drug resistance in ovarian cancer cells. The results I have procured in recurrent ovarian cancer cell (ROCC) lines show a marked overexpression of FAS in human ovarian tumor samples and resistant ovarian cancer cell lines to be correlated with decreased apoptosis and increased cell migration. In addition, the FAS inhibitor cerulenin, in combination with cisplatin, causes significant induction of apoptosis and migratory inhibition in drug-resistant ovarian cancer cells. Therefore, I hypothesize that a mechanism by which ovarian cancer cells develop drug resistance is by upregulating the FAS pathway, allowing the increased metabolic demands of the cancer cells to be met. This pilot study suggests that targeting molecular FAS using a small molecule inhibitor may have therapeutic potential for treating drug-resistant ovarian cancer.

Background:

Ovarian cancer

Amongst all of the gynecologic diseases, ovarian cancer is the leading cause of death with an average survival of five years after diagnosis and an estimated malignancy rate of just under 70%, according to the Foundation for Women's Cancer. Due to the common late-stage diagnosis of ovarian cancer, tumor resection followed by taxane- or platinum-based adjuvant combination chemotherapy treatment is standard protocol; however, around 75% of patients relapse within two years of treatment and qualify for treatment of tumor recurrence (1, 2). Two common chemotherapeutic agents used in combination therapy are paclitaxel, a taxane-based chemotherapeutic, and cis-diaminedichloro platinum II (CDDP) (**Figure 1**), a platinum-based chemotherapeutic. A problem that routinely arises in relapsed patients is the development of a platinum-resistant recurrent tumor, thus begetting a narrowed scope of effective therapeutic options. Consequently, a mechanistic understanding of chemotherapy resistance in ovarian cancer is pivotal in the advancement toward a cure.

Hypothesis: Our hypothesis is that increased expression of FAS in ovarian cancer plays a central role in the development of resistance to anticancer therapy, and that targeting the FAS protein using small-molecule inhibitors will increase treatment success, regardless of cellular phenotype or tumor microenvironment. This will lead to decreased disease progression and improve patient prognosis.

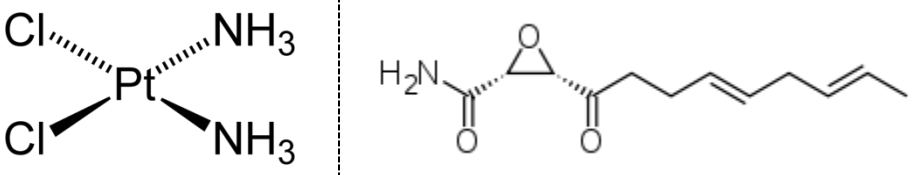


Figure 1: Cisplatin (CDDP) and Cerulenin, respectively. CDDP inhibits cancer growth by forming DNA adducts in target cells. Cerulenin acts to prevent cancer proliferation via inhibition of the N-terminal catalytic domain of FAS.

Acquired drug resistance in recurrent ovarian cancer: a threat to successful treatment

Acquired drug resistance remains a major challenge in the clinical treatment of ovarian cancer. Cancer cells that survive exposure to common chemotherapy agents will develop a resistance to subsequent intervention (1) and the overall clinical response in chemotherapy-treated patients with recurrent ovarian cancer is only 40–60% (1,2). Therefore, the investigation and development of potential new therapeutic compounds is necessary in overcoming CDDP-mediated drug resistance in recurrent ovarian cancers.

Fatty acid synthase

FAS is a metabolic enzyme involved in the synthesis of long-chain saturated fatty acids that are essential for membrane synthesis in proliferating cells. The overexpression of FAS is a recently-discovered phenomenon found in most ovarian cancer tissues, and the increased expression of this enzyme is associated with increased aggressiveness and poor patient survival. Increased expression of FAS occurs very early in ovarian cancer development and is more pronounced as the tumor progresses toward more advanced stages (4-6). As a result, FAS has emerged as a potential tumor marker for breast cancer and endometrial cancer, as well as a potential therapeutic target. We recently found that FAS is highly expressed in various human ovarian cancer cells and human tumor specimens, particularly in early stage disease (**figure 3**). Thus, it is logically supported that FAS expression may have potential as a prognostic indicator of ovarian cancer.

Biological function of FAS

Important in the regulation of endogenous fatty acid, cholesterol, and phospholipid synthesis, the SREBPs are a family of membrane-bound transcription factors located in the rough endoplasmic reticulum. There are three isoforms of SREBP known: SREBP-1a and SREBP-1c, which are generated from alternative splicing of the same gene on chromosome 17, as well as SREBP-2, which is located on chromosome 22 (3). When the cellular environment is deprived of lipid or sterol molecules, these SREBPs are activated in a proteolytic fashion by the SREBP-cleavage activating proteins (SCAP), which allows the activated SREBP to translocate into the nucleus and begin transcribing its target genes (3).

Among the genes transcribed, the most notable are those involved in cholesterol biosynthesis, the modulation of low density lipoprotein (LDL) membrane receptors, which operate in a negative-feedback loop, and those involved with fatty acid uptake and synthesis (FAS, acetyl CoA carboxylase, lipoprotein lipase) (3). With regard to cholesterol regulation, SREBPs upregulates LDL receptor production, which bind and endocytose extracellular cholesterol when endogenous cholesterol synthesis is not adequate to meet the cell's metabolic needs; however, when the intracellular environment is saturated with cholesterol, each SREBP-encoding gene begins to cotranscribe miRNAs for its respective mRNA, effectively inhibiting translation (4). First implicated in the regulation of fatty acid synthesis in 1993, the SREBP pathway is now considered to be one of the master regulatory elements of the FAS pathway, however regulation of FAS independent of SREBP has also been observed.

Essential for the survival of cancer cells, FAS mediates *de novo* lipogenesis and the production of long-chain fatty acids, specifically palmitate, from precursor molecules procured from normal glucose metabolism, specifically citrate, acetyl-coenzyme A (CoA), and malonyl-CoA (5, 6). Through a series of enzymatic reactions, the carbohydrate precursors are converted into fatty acids, glycerol, and phospholipids utilized for biosynthesis of endogenous signaling molecules and components of the cytoplasmic membrane (5). In a typical cellular environment, fatty acids are not required in large quantities and, therefore, are not commonly synthesized endogenously; healthy cells are able to rely on the exogenous uptake of fatty acids to self-sustain growth (7). However, with the markedly increased metabolism of a tumor microenvironment, cancer cells often exhibit a shift from exogenous uptake of fatty acids to endogenous production of these molecules utilizing FAS (8). By producing endogenous fatty acids using FAS, the rapidly-proliferating cancer cells are able to meet the elevated demand for biosynthetic molecules needed for expanding the cytoplasmic membrane and for subsequent division. Intracellular production of long-chain fatty acids also provides a source of energy via β-oxidation of lipids into metabolism precursor molecules, such as acetyl-CoA, NADH, and $FADH_2$. Consequently, FAS plays a pivotal role in maintaining metabolism, proliferation and division pathways of cancer cells.

Within the field of medicine, the development of targeted therapies is a rapidly-expanding topic of interest and recent research suggests FAS as being a potential therapeutic target for inhibition of tumor growth (6). Inhibitors of FAS have been shown to induce apoptosis in cancerous cell lines *in vitro*, to delay both benign and malignant tumor growth *in vivo*, and to decrease the rate of formation of ascites *in vivo*; however, the mechanisms of action of many of these inhibitors are poorly understood (6). One inhibitor in particular whose mechanism has been delineated, cerulenin, has provided promising direction regarding the future of targeted FAS inhibition by demonstrating specific intratumor activity within just 6 hours of administration *in vivo*. In addition to demonstrating effective bio-absorption *in vivo*, Cerulenin is also shown to be selectively cytotoxic to cancer cells with an increased demand for fatty acid biosynthesis as opposed to non-cancerous cells *in vitro* (9).

<u>Inhibiting ovarian cancer proliferation and migration</u>

Cerulenin (2,3-Epoxy-4-oxo-7,10-dodecadienoylamide) (**Figure 1**), an early, small-molecule inhibitor of FAS, is a common means by which the role of FAS is studied in the laboratory. Of the six catalytic domains of FAS, the N-terminal β-ketoacyl synthase (βKS) domain is most commonly targeted by FAS inhibitors, including cerulenin (10, 11). The structure of cerulenin includes an epoxide as its

active functional group which inhibits the FAS βKS activity. The instability of the epoxide allows the cerulenin to attack the active site of βKS and covalently binds to the sulfur on the Cysteine 1305 residue on the FAS protein (12). The βKS domain of FAS is typically orchestrates a condensation reaction between acetyl-CoA and malonyl-CoA and catalyzes the formation of the fatty acid palmitate, which can be metabolized and used in various biosynthetic reactions (13). Due to this inhibition of the βKS catalytic domain, palmitate synthesis and subsequent metabolism and energy production are discontinued (6, 13).

In addition to serving as a means by which cancer cells are able to meet increased metabolic demands, FAS has been implicated in mediating chemotherapy resistance within cancer cells, though the mechanism remains largely ambiguous. CDDP is a platinum coordination compound with a square-planar geometry, which was initially investigated as a cancer chemotherapy in 1965 when it was found to inhibit cell division in E. coli (14). CDDP enters cells via transport proteins (15) or passive permeation through the cytoplasmic membrane and induces apoptosis in cancer cells by targeting the nucleus upon entering the cell and forming strong intra- and inter-strand DNA adducts, effectively preventing transcription and replication (16, 17). While being a treatment option for an array of cancers, one of the major obstacles to CDDP being an effective therapy is the development of chemoresistance in its target cancer cells. This resistance to platinum agents has been attributed a few different mechanisms: reduced intracellular drug accumulation, whether due to reduced cisplatin uptake or increased cisplatin efflux (18-20); glutathione-mediated intracellular cisplatin inactivation and detoxification (21); and increased DNA repair by nucleotide excision repair (22). Dirk O Bauerschlag et al. demonstrated that FAS expression at both the protein and mRNA levels in cancer cells was up to 100-fold that of the FAS expression in healthy cells and, as predicted, when treating both CDDP-resistant and -sensitive cell lines with a treatment regimen of CDDP, higher rates of apoptosis and decreased cell viability were observed in sensitive cells while the opposite was observed in resistant cells.

The information presented above has inspired the current project, which is to not only investigate the mechanism underlying cisplatin resistance in ovarian cancer, but to also explore the pathway in relation to FAS; these preliminary *in vitro* wound-healing, western blotting, annexin V (apoptosis assay), and *ex vivo* immunohistochemistry (IHC) results have been verified in replicates. Based upon previous research and the current results, I hypothesize that FAS not only plays a substantial role in the development of resistance to anticancer therapy in ovarian cancer cells, increasing the

survivability of cancer cells, but it also acts to inhibit the mechanism by which CDDP induces apoptosis, directly or indirectly. While the mechanism is not explored in depth in this paper, these results provide promising support that FAS impedes upon the CDDP-mediated apoptosis pathway in ovarian cancer cells.

Methods:

Immunoblot

TR127 cells, a ROCC line, were cultured to 60% confluence in supplemented RPMI medium (10% FBS, 1% PS, 1% sodium pyruvate) and arbitrarily placed into one of eight groups: control, CDDP 10μM treated, cerulenin 20μM treated, and CDDP 10μM + cerulenin 20μM concomitant treatment; or control, CDDP 5μM, CDDP 10μM, and CDDP 20μM. After 24 hours of treatment, the cells were collected via scraping and centrifuged at 1,200 RPM, the pellet was subjected to 30 minutes of lysing (200 μL, TN1 lysis buffer with 1x ThermoFisher Halt protease inhibitor [EDTA 0.005 mM, AEBSH HCL 1mM, aprotinin 0.8 mM, bestatin 0.05 mM e-64 0.015 mM, leupeptin 0.02 mM, pepstatin A 0.01 mM]) and was centrifuged at 10,500 RPM. The protein was collected and quantified using ThermoFisher Pierce protein assay kit (catalog: 23225) and a spectrophotometer reading absorbance at 562 nm. After quantification, 30μg protein of each sample were added to sample buffer and denatured at 98°C for 8 minutes. The denatured samples were loaded into BioRad mini gels (catalog: 4568081) and ran under denaturing, SDS-containing conditions at 100 V for 2 hours. The protein was transferred from the SDS gel onto a nitrocellulose membrane in 1x tris-glycine buffer and 20% methanol at 100V for 2 hours. The resulting proteins were analyzed using an HRP-detecting chemi-luminescence kit (GE, catalog: RPN2232) and developed using autoradiographic film (Denville, catalog: E3018) in an x-ray developer.

Migration

100,000 TR127 cells were seeded into 6-well plates in supplemented RPMI medium. Once the cells reached 95% confluence, a 10μL cell culture tip was used to scratch three lines in each well, the existing medium was removed, and the wells were labeled accordingly: zero hour (RPMI), 24-hour control (RPMI), CDDP 5μM, CDDP 10μM, CDDP 20μM, cerulenin 20μM, CDDP 10μM + cerulenin 20μM. 2mL of RPMI medium containing each treatment was added to its respective well. Pictures were immediately taken of the zero-hour (RPMI) control scratches. After 24 hours elapsed, pictures of the 24-hour positive control, CDDP 5μM, CDDP 10μM, CDDP 20μM, cerulenin 20μM, and CDDP 10μM + cerulenin 20μM were taken. The wound areas remaining after 24 hours of treatment were

quantified using ImageJ software. Each measurement was taken in triplicate. Results are reported as the inverse of the average area remaining with standard deviation calculated.

IHC

Ex vivo CDDP-sensitive and -resistant high-grade, serous, ovarian carcinoma tumor tissues were collected from human patients with IRB approval. The tissues were embedded in optimal cutting temperature (OCT) medium (Sakura, catalog 4583) and stored at -80°C until sectioning. Sectioned slides (8μm thickness) were then obtained following previously-described methods(23).

Annexin V apoptosis analysis

TR127 ROCC lines were cultured to 65% confluence in 10mm plates in RPMI medium and subsequently exposed to one of the following treatments: CDDP 10μM, cerulenin 20μM, or combination treatment. After 24 hours, apoptotic cells were measured via flow cytometry (EPICS Profile II flow cytometer; Coulter Corp., Hialeah, FL) according to the ThermoFisher Annexin V staining protocol (catalog: V13241).

Results:

FAS/FAK expression in ovarian cancer:

Figure 2 shows a comparison of basal levels of FAS and FAK in a cisplatin-sensitive (CS) cell line relative to those in cisplatin- (CR) or multi-drug-resistant (MDR) cell lines run in our lab. In addition to these *in vitro* results, *ex vivo* fluorescent and non-fluorescent IHC results performed using CDDP-sensitive and -resistant human ovarian tumor tissues revealed a significant correlation between FAS expression level and cisplatin resistance. As

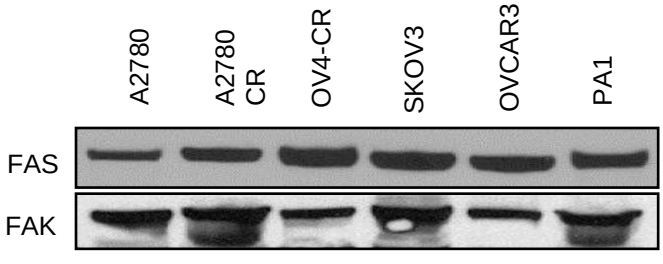


Figure 2: Basal, endogenous FAS (and FAS-activated FAK) expression in drug-resistant human ovarian cancer cell lines (25).

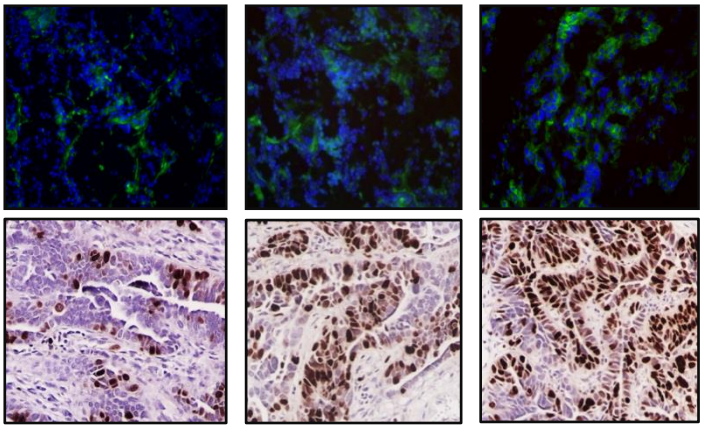


Figure 3: FAS (green, brown) in cisplatin-resistant ovarian tumors compared to that of cisplatin-sensitive tissue. CS: (p=0.0025) CR: (p=0.0156) (N=6 CS, N=4 CR).

seen in **figure 3**, sensitive tissues (upper and lower left) exhibit much lower FAS expression (green and brown staining) as compared to those sections taken from resistant tissues (four remaining images).

Cisplatin treatment enhances FAS/FAK and its target genes

The CDDP optimization treatments demonstrated the ideal concentration of CDDP as being 10μM, which corresponds with the recommended clinical dose. When subjected to either 5μM, 10μM, or 20μM, and collected for protein analysis, TR127 cells express the highest levels of FAS, cyclin D2, survivin, focal adhesion kinase (FAK), survivin, cyclic myc, vascular endothelial growth factor (VEGF), and matrix metallopeptidase 2 (MMP2) when treated with 10μM CDDP as compared to the control, untreated cells (**figure 4**). Western blotting results have consistently found that with the treatment regimens, the levels of apoptotic proteins consistently increased in this order (from lowest measured apoptosis to highest measured apoptosis): control, CDDP 10μM, cerulenin 20μM, CDDP 10μM + cerulenin 20μM; while demonstrating increasing FAS expression in this order: cerulenin, CDDP/cerulenin, control, CDDP (**figure 5**).

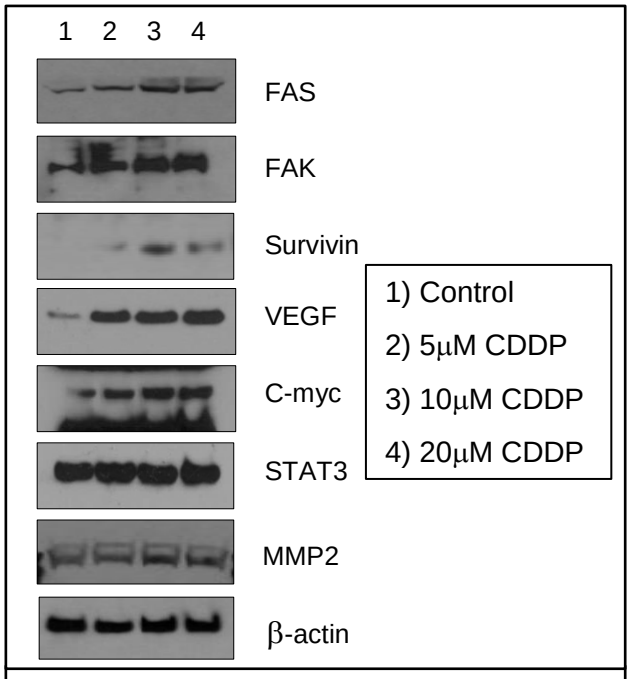


Figure 4: Western blot using TR127 cells to study the effect on expression following treatment with CDDP, the chemotherapeutic agent to which TR127 cells are resistant. Notable increase in FAS and FAK (both involved in migration), as well as survivin (protects cells proliferation), and MMP2 (involved in cell invasion).

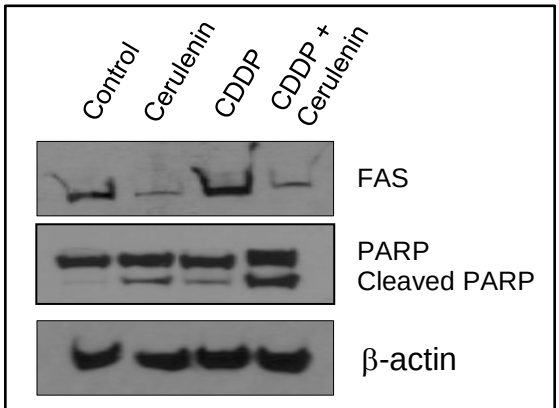


Figure 5: Western blot using TR127 cells to study the effect on expression of FAS (metabolic activity and migration) and cleaved PARP (involved in apoptosis) following treatment with CDDP, the chemotherapeutic agent to which TR127 cells are resistant; or cerulenin, the FAS inhibitor.

Targeting FAS via small molecule inhibitor

Visually and quantitatively, *in vitro* wound-healing assays also showed that with an increase in CDDP concentration, there was an increase in migratory ability. When the concentration was increased from 0 µM to 5 µM to 10 µM, optimal concentration of CDDP was found to be 10µM, which is equivalent to the translated clinical dose. However, when cerulenin, the FAS inhibitor, was added concomitantly the cells, both cell viability and migratory ability were decreased (**figure 6**). Further, confirmation of these effects of FAS inhibition by cerulenin can be seen in a previous publication that was also published by our lab; however in place of cerulenin, siRNA was used to inhibit FAS expression (24).

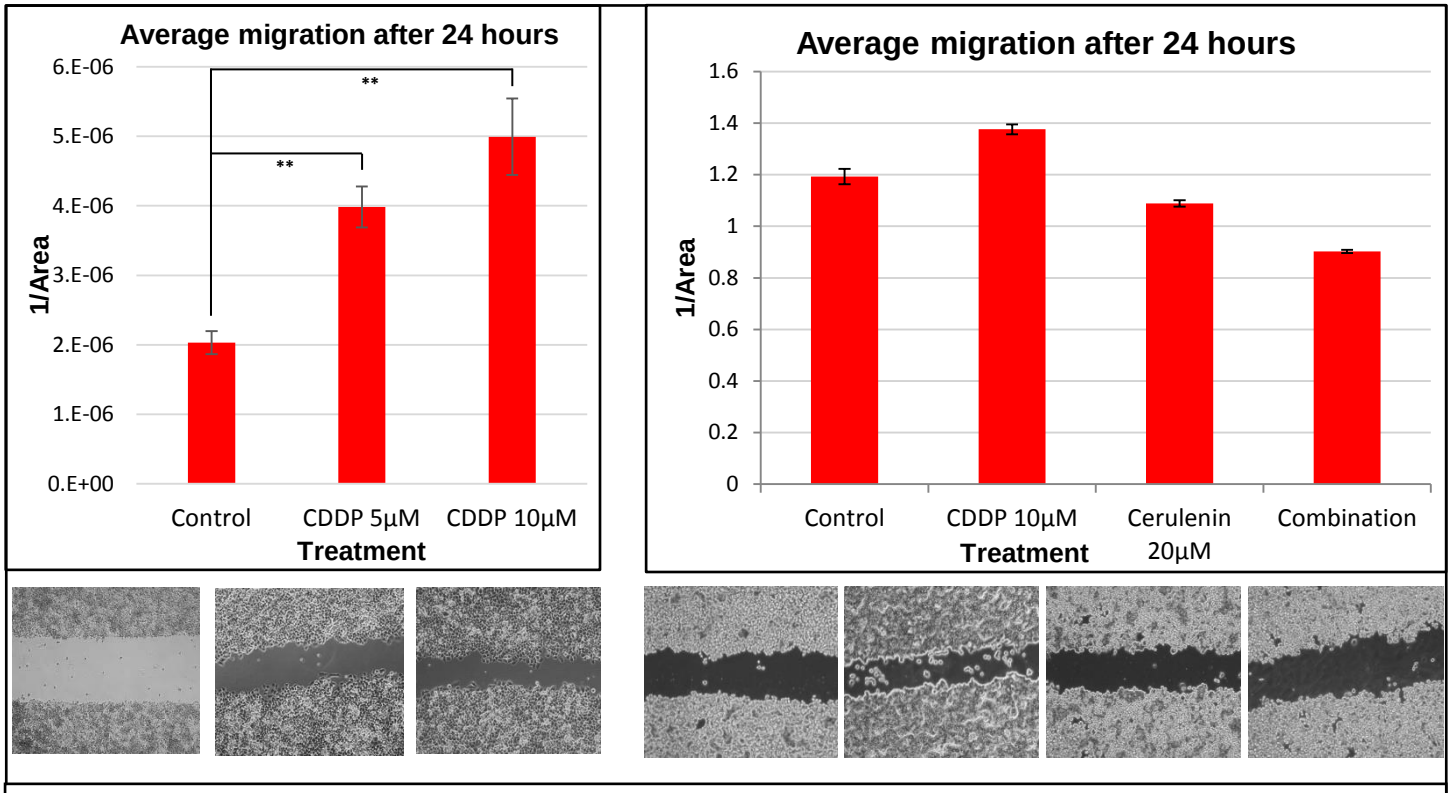
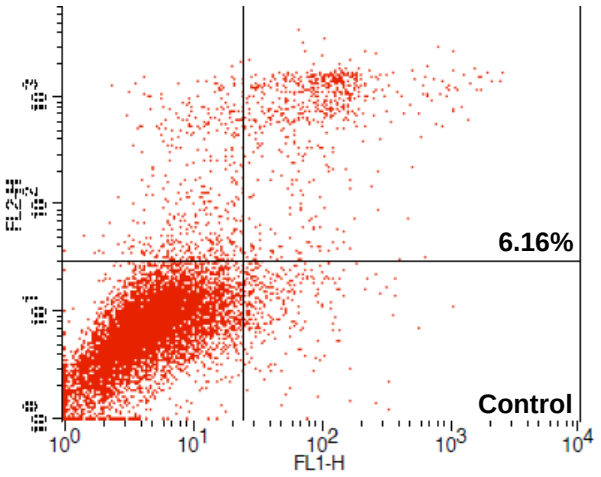


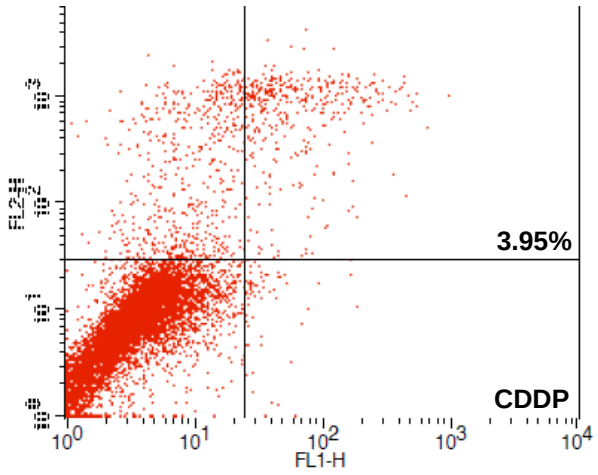
Figure 6: (**Upper Left**) This bar graph shows the average migration of TR127 cells after a 24-hour wound-healing assay as a measurement of the inverse of the wound area remaining after 24 hours of incubation; normalized to the 0-hour negative control. (**Lower Left**) An example of each group from the assay: positive control, 5µM CDDP, 10µM CDDP, respectively.
(**Upper Right**) This bar graph shows the average migration of TR127 cells after a 24-hour wound-healing assay as a measurement of the inverse of the wound area remaining after 24 hours of incubation; normalized to the 0-hour negative control. (**Lower Right**) An example of each group from the assay: positive control, 10µM CDDP, 20µM cerulenin, 10µM CDDP + 20µM cerulenin, respectively.

Evaluating apoptosis

Analysis of cellular apoptosis was carried out using annexin V and propidium iodide stains. TR127 cells were cultured in 10cm culture plates, exposed to 24 hours of treatment and then subjected to a combination of annexin V (tags live cells) and propidium iodide (tags dead, or late apoptotic cells).

The flow cytometry results even further supported our existing results that FAS plays a pivotal role in mediating CDDP resistance, as shown in **figure 7**.



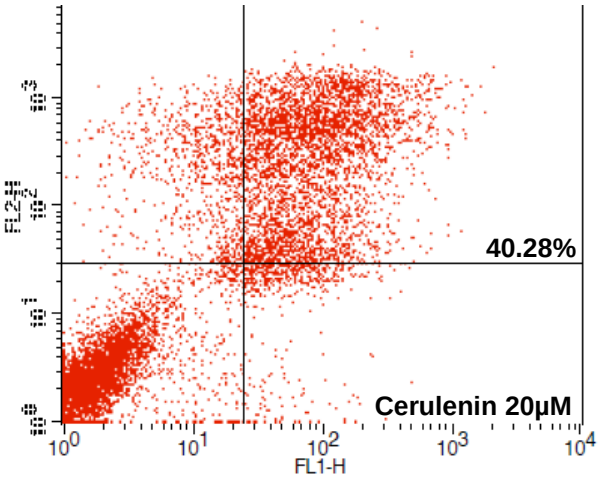


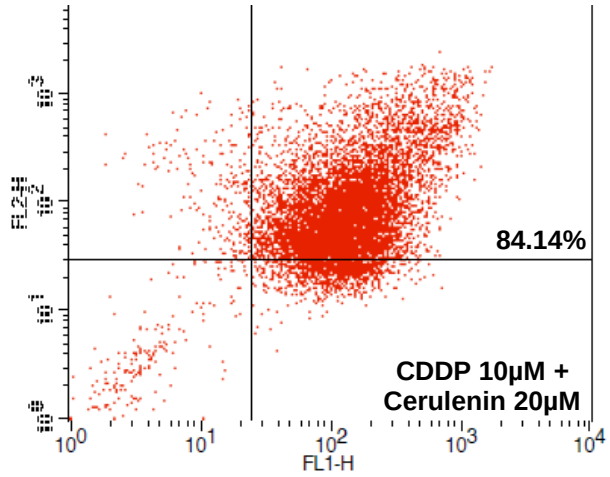
Control

Quad	Events	%Gated	%Total
UL	416	4.16	3.98
UR	616	6.16	5.89
LL	8676	86.76	82.90
LR	292	2.92	2.79

CDDP 10μM

Quad	Events	%Gated	%Total
UL	560	5.60	5.39
UR	395	3.95	3.80
LL	8981	89.81	86.41
LR	64	0.64	0.62





Cerulenin 20μM

Quad	Events	%Gated	%Total
UL	866	8.66	6.87
UR	4028	40.28	31.96
LL	4595	45.95	36.46
LR	511	5.11	4.05

CDDP 10μM + Cerulenin 20μM

Quad	Events	%Gated	%Total
UL	243	2.43	1.79
UR	8414	84.14	61.99
LL	182	1.82	1.34
LR	1161	11.61	8.55

Figure 7: Annexin V results obtained from TR127 cells cultured with RPMI medium (positive control), 10 μM CDDP, 20 μM cerulenin, or 10 μM CDDP + 20 μM cerulenin. The lower left quadrant represents viable, healthy cells. The upper left quadrant represents the percentage of necrotic cells stained with both annexin V and propidium iodide. The lower right quadrant represents cells beginning early apoptosis that are stained with only annexin V and the upper right quadrant represents cells already having undergone apoptosis, which are stained with only propidium iodide.CDP-treated cells underwent less apoptosis (3.95%) than did the control cells (6.16%); the combination treatment cells underwent significantly more apoptosis (84.14%). Results are shown as percentages that are averages of triplicates.

Cumulatively, these results demonstrate that levels of FAS are positively correlated with treatment with CDDP (up to 10μM) and that increased FAS is positively correlated with increased cell viability. While no mechanism has been specifically explored, the results support my hypothesis that FAS takes part in mediating CDDP resistance in ROCCs consequent of the upregulation of FAS stimulating one of the four resistance pathways mentioned earlier in this report, while simultaneously allowing the cell to meet its newfound metabolic demands.

Discussion

Fatty acid synthase is identified as the oncogenic antigen-519 (25) and it is now clear that FAS is highly expressed in a biologically aggressive subset of various human tumors, including ovarian cancers (26). Endogenous FAS expression is extremely low in most normal human tissues, except lactating breast and cycling endometrium (5). Recent evidence shows that high levels of FAS are found to correlate with poor prognosis and survival for various cancers, including ovarian cancer (27, 28).Cerulenin, a natural product, was the first specific inhibitor of FAS to be studied (29) and has been evaluated in a variety of human cancer cell lines and xenograft tumors. Current research is now highlighting inhibitors of the FAS pathway, such as cerulenin, as a potential drugs for targetted anticancer therapy.

Drug resistance is a major clinical obstacle in creating a successful ovarian cancer chemotherapy. Recent research *in vitro* and *in vivo* suggests multiple molecular mechanisms of resistance identified in ovarian cancer, such as ABC transporter-mediated drug efflux and hypoxia-mediated oncogene activation (30). In addition, our pilot study identified a similar correlation between drug resistance and overexpression of fatty acid synthase in human tumor samples, as that seen in the resistant ovarian cell lines. Further, this study showed that overexpression of FAS is indeed correlated with drug resistance, inhibition of apoptosis, and and decreased migration in ovarian cancer cell lines.

The molecular mechanism of FAS-mediated drug resistance in ovarian cancer is not yet known and the relationship between drug resistance and overexpression of FAS in ovarian cancer cells is poorly understood. Based upon previous research, FAS overexpression in drug resistant ovarian and breast cancer cells causes an overproduction of palmitate to increase resistance to anti-cancer drug-induced apoptosis (30). Another study showed that fatty acids synthesized by FAS in cancer cells are saturated and monounsaturated and, therefore, the increased lipid synthesis in cancer cells may change both the quantity of membrane lipids required for cell growth as well as the lipid composition of the membrane (31).

Conclusion

Drug resistance in is a major impediment that prevents effective treatment of the leading cause of death amongst women, ovarian cancer. Resistance, specifically platinum resistance, is most often seen in recurrent ovarian cancer tissues and has been attributed to reduced cisplatin uptake or increased cisplatin efflux, glutathione-mediated cisplatin inactivation and detoxification, and increased DNA repair by nucleotide excision repair. FAS has been implicated in CDDP resistant ovarian cancer, is consistently found to be upregulated in these tissues, and is positively correlated with increased migratory ability and resistance to apoptosis. Our results support these ideas, showing that with increased CDDP exposure (up to 10μM) there is a concurrent increase in intracellular FAS mRNA and protein expression. Additionally, it is shown that when the 10μM dose, which normally contributes to increased migration and decreased apoptosis, is coupled with a small-molecule inhibitor or FAS, the cells undergo significant stress and eventually apoptosis. While the mechanism underlying the interaction between CDDP and FAS remains largely undefined, these results support the idea that FAS mediates resistance to common anticancer treatments.

Ongoing and future direction

Moving forward, we will use TR127 and TR182 (also a platinum-resistant line) cells to explore a more specific mechanistic understanding of the role FAS plays in mediating platinum resistance. Western blotting will be used to analyze upstream and downstream protein levels in the FAS pathway to allow us to confirm that FAS is indeed the main driving component behind CDDP resistance in ovarian cancer cells. Wound-healing assays will be reconfirmed and optimized by pretreating the TR127 cells with mitomycin C. This will allow us to specifically identify the effect of CDDP and cerulenin on cell migration by separating proliferation from migration. In addition, immunocytochemistry will be used to confirm differential FAS levels in various CDDP treatment concentrations as well as in those cells treated with cerulenin. Once this *in vitro* work is complete, orthotopic mouse models of ovarian cancer will be created to study the effect of cancer progression as a result of these treatments. Together, the results procured from these experiments will help us to confirm the potential of FAS being an effective target for anticancer therapy.

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