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INVESTIGATION OF P-GLYCOPROTEIN (PGP) INDUCTION BY PGP SUBSTRATES TO
INDUCE PACLITAXEL RESISTANCE IN OVARIAN CANCER CELLS

by

RYKER PENN

A THESIS

Presented to the Faculty of the University of the Incarnate Word
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

UNIVERSITY OF THE INCARNATE WORD

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Ryker Penn

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Ryker Penn

University of the Incarnate Word, 2019

The purpose of this study was to investigate the development of chemotherapeutic resistance to paclitaxel in ovarian cancer cells after treatment with drugs that are substrates for P-glycoprotein (PGP). A core concept of this experiment was to identify if PGP substrate drugs could also act as PGP inducers after prolonged treatment in SKOV-3 ovarian cancer cells. In order to test this, SKOV-3 cells were exposed to either fexofenadine, a PGP substrate used as an antihistamine, or the chemotherapeutic drug vinblastine. After 42 days of drug treatment, *ABCB1* gene expression was measured by qRT-PCR. Analysis of *ABCB1* expression in treated cells revealed that fexofenadine was unable to significantly induce gene expression in SKOV-3 cells. Although some cells treated with vinblastine did exhibit some significant increases in *ABCB1* expression, vinblastine exposure overall could not reliably induce *ABCB1* gene expression within SKOV-3 cells. After testing for PGP induction, treated cells were exposed paclitaxel and tested for cell survivability. The results indicated that cells with induced PGP exhibited reduced survivability against paclitaxel exposure.

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Ovarian Cancer affects around 204,000 women a year all over the world and of those it is responsible for nearly 125,000 deaths. In the United States alone approximately 22,000 new cases manifest each year leading to more deaths than any other type of female reproductive cancer (12). These statistics are bolstered by the fact that nearly 70% of ovarian cancers are diagnosed at an advanced stage the result being that women with this diagnosis can at best only expect to survive for another 5 years. This poor diagnosis has led to this form of cancer being known as a 'silent killer' as it shows very few symptoms until after it metastasizes within the peritoneal cavity of the patient (2). Similar to other cancers which are often detected in advanced stages, cancerous cells often develop drug resistance which renders numerous chemotherapeutics largely ineffective against the disease and forces doctors to treat patients with other medications that may prove less effective (2).

The development of this resistance within cancerous tissues to chemotherapeutic drugs is known as multidrug resistance (MDR) and it represents a grave threat to the efficiency of most widely used chemotherapy agents (15). It was discovered nearly 40 years that MDR played a vital evolutionary role in an animal's defense against environmental toxins by mobilizing cellular efflux pumps present on the cell membrane to pump out invading toxic substances (19). The overexpression of these pumps in cancerous cells however is what causes this normally advantageous mechanism to work against the greater good of a cancerous body essentially rendering many chemotherapeutic drugs ineffective. A significant mechanism which confers resistance to cancer is their active removal of the chemotherapeutic drugs from the cancer cells by drug transporters in the ATP Binding Cassette (ABC) family. Those that have been found to be associated with MDR include P-glycoprotein (PGP), Multidrug Resistance Proteins (MRPs),

and Breast Cancer Resistance Protein (BCRP) but this proposed project will focus specifically on the role of PGP in drug resistance (15).

PGP is a 170-kDa ABC transporter comprised of 1280 amino acids which acts as an ATP energy dependent efflux pump. It is encoded by the *ABCB1* gene, alternatively known as *MDR1*, which is present on chromosome 7q21 (3). It has 2 symmetrical amino and carboxyl halves known as cassettes which consist of 6 transmembrane domains (TMDs) each. These TMDs are the channels by which cells utilize these binding cassettes to power the expulsion of cytotoxic drugs out of the cell to by hydrolyzing ATP. This action effectively reduces intracellular drug concentrations. The ABCs are linked together by a polypeptide loop of about 80 amino acids with an ATP-binding motif (3). There are 2 ATP binding domains (alternatively known as Nucleotide Binding Domains) which power PGP function and they are both located in the cytoplasm and function to transfer energy to transport substrates across the membranes. Both NBDS are essential for the ability to bind drugs to the binding sites located on the extracellular surface of the protein (18). These drug binding sites are known to be quite mobile and likely adjust based on different drugs employing a mechanism known as ‘substrate-induced fitting’ (1). This flexibility is what allows them to be involved in so many processes of the body and to have effects against such a diverse array of drugs with dissimilar structures (3).

PGP is expressed in the lining of the gastrointestinal tract and endothelial cells of the blood brain barrier. It is also present in many other tissues including the bile duct, adrenal gland, kidney tubules, small intestine, pancreatic duct, heart, lungs, spleen, certain skeletal muscles, and the ovarian blood barrier (4). PGP functions by pumping the drugs out of the cells after oral ingestion due to its station in the apical membranes of enterocytes of the intestine. Then when the drugs enter systemic circulation, PGP aids in the removal of these drugs through urine and

bile due to its presence on hepatocytes and the apical surface of the kidney's tubular cells (4).

PGP also appears to regulate hormone distribution and may regulate cell differentiation, proliferation, immune responses and programmed cell death otherwise known as apoptosis (1).

In healthy cells, P-glycoprotein mediated drug efflux is believed to occur based on one of three possible mechanisms. The first is known as the classical pore pump model and it has PGP forming a hydrophilic tunnel by which recognized substrates are exchanged from the cytosol into the extracellular media. The second method is called the hydrophobic vacuum cleaner model and it has PGP binding directly with hydrophobic substrates within the inner side of the plasma membrane and then expelling them out of the cell upon identifying them as xenobiotic in nature. The substrates are held between the internal aqueous compartment and the inner membrane surface of the cell before PGP makes contact which then results in conformational alterations to the transporter itself to remove the substrate to the external medium (5). The third and final model is the most accepted of the three, it is called the flippase model and it has PGP intercepting the drug as it travels through the lipid membrane and flipping the drug from the inner leaflet (the inner side of the plasma membrane) toward the outer leaflet (the outer side of the plasma membrane) against the concentration gradient via ATP hydrolysis (18).

There are four primary mechanisms of drug resistance. The first is enhanced efflux of a drug by proteins such as the aforementioned ABC family of efflux pumps including P-glycoprotein. Other efflux transporters include the Breast Cancer Resistance Protein and the Multidrug Resistance Proteins 1-3. The second method of drug resistance is through the modulation of drug-induced apoptotic progression in which PGP may also be involved. The third method involves the development of changes in the cell's ability to fix DNA damage due to genetic variance in the coding for repair proteins of drug-targeted molecules (1). The fourth and final

method is the acceleration of drug metabolism by enzymes such as glutathione S-transferase or GST. It is generally accepted that MDR is most often the result of multiple of the above methods occurring together but the most prevalent method associated with multidrug resistance is the enhanced efflux by ABC family pumps (1).

In addition to the methods above, there are some unique chemotherapeutic drugs which cannot be transported by PGP whose use can result in the manifestation of the MDR phenotype through other more unconventional means. In the unique case of cisplatin-induced PGP expression, the drug can act as a ligand to the xenobiotic detecting pregnane X receptor (PXR) which has been shown to have a degree of transcriptional control over PGP (1,16). In this way, the development of drug resistance to cisplatin in cancer cells is an indirect method of resistance to PGP rather than a direct link to the pump itself (6, 15). This PXR-mediated PGP overexpression has further been associated with resistance to both paclitaxel and vinblastine in LS180 colon cancer cells (9).

In cancerous cells, drug resistance conferred by the expulsion of drugs from the cells by ABC transporters such as PGP, BCRP, and numerous other MRPs. The overexpression of PGP can greatly diminish the intracellular retention of these therapeutic agents (15). In recurrent ovarian cancer cases the changes in *ABCB1* expression is assessed as PGP has been proposed as a viable biomarker for drug resistance in cancer patients (22). Further, induced PGP expression has been detected in tumors exposed common drugs which act as immunosuppressants, antihypertensives, antihistamines, antimicrobials, and anti-inflammatories (16). Due to the high diversity of drugs that are recognized and transported by PGP however there are also many therapeutic drugs which are known to act as both PGP substrates and inhibitors which include

calcium channel agonists, calmodulin antagonists, local anesthetics, steroids, protein-kinase inhibitors, detergents, and other immunosuppressive agents (1).

The most efficacious chemotherapeutic drugs used in the treatment of ovarian cancer today are cisplatin, its analogue carboplatin, and paclitaxel which all are commonly administered together in a treatment regimen (12). An issue with these treatments however is that resistance may develop to these drugs, ultimately leading to treatment failure. While cisplatin can develop resistance using the unique PXR-related methodology mentioned earlier, cancer cell resistance to paclitaxel has primarily been found to be mediated through ABC proteins such as PGP (15). Another anti-cancer chemotherapeutic drug of interest is vinblastine which is a vinca alkaloid used as a cytotoxic agent to treat patients with lymphoma, leukemia, ovarian cancer, breast cancer, colorectal cancer, as well as other types of solid tumors. Like paclitaxel, vinblastine is a known PGP substrate which has been shown to be capable of inducing PGP (9). The common antihistamine fexofenadine (commercially known as Allegra) is also a known PGP substrate which also may be capable of PGP induction (personal communication, Dr. Christopher Farrell).

In recent years however, research has been done using cancer cell lines to identify drugs which can act as substrates for PGP. These medications that are commonly used by patients for co-morbidities may cause an induction of PGP expression in cancer cells, possibly conferring resistance to chemotherapeutics used to treat those tumors. The purpose of this experiment was to induce the expression of PGP in the ovarian cancer cell line SKOV-3 using the PGP substrates fexofenadine and vinblastine and then to determine if the PGP-induced SKOV-3 cells display primary drug resistance to paclitaxel. We hypothesize that when ovarian cancer cells are exposed to these PGP-substrate drugs the cancer cells will begin to overexpress the P-glycoprotein transporter and will then display drug resistance.

Materials and Methods

Reagents

The drug vinblastine acted as a positive control and fexofenadine served as the test PGP inducer, both were purchased from Sigma Aldrich. The chemotherapeutic paclitaxel used to determine drug resistance in PGP expressed cancer cells was purchased from Sigma Aldrich. The SKOV-3 human ovarian cancer cell line and RPMI 1640 medium used to culture them were purchased from ThermoFisher Scientific along with 10% Fetal Bovine Serum. When sub-culturing cells, Trypsin 0.53 mM EDTA solution was purchased from ATCC and used to disperse cell layers before the new medium is added. In preparation for performing quantitative real-time PCR (qRTPCR), the Purelink RNA Mini kit was purchased from ThermoFisher for RNA isolation along with the SuperScript II Reverse Transcriptase kit for cDNA synthesis from Invitrogen. Taqman Advanced Master Mix and custom assays for *HPRT1* and *ABCBI* were purchased from ThermoFisher Scientific. Additionally, dNTPs and random hexamer primers for expression profiling were purchased from Invitrogen. To determine cell viability the PrestoBlue Cell Viability Assay kit was purchased from ThermoFisher Scientific.

Cell Culture

The SKOV-3 cells were maintained as monolayers in complete RPMI 1640 medium with 10% fetal bovine serum. SKOV-3 cells were grown for three passages before beginning any drug exposures. After the third passage, all cells were given at least seven days of growth in the incubator with media changeout at least twice per week depending on cellular health. The cells were contained in either T.75 flasks or 75mm petri dishes and stored in a 151L CO₂ incubator set to 5% and 37°C.

Dose Response Curve

To determine drug concentrations of fexofenadine and vinblastine for use in the induction studies which could maintain cellular health, a dose response curve was performed using separate groups of SKOV-3 cells. Each group was treated with one of three concentrations of either vinblastine or fexofenadine. Vinblastine and Fexofenadine were solubilized in DMSO first and then diluted into water. Vinblastine was prepared at concentrations of 2nM, 10nM and 30nM with in a final DMSO concentrations of 4.732e-7%, 1.23e-5%, and 0.0001% respectively. Fexofenadine was prepared at concentrations of 25µM, 75µM, and 125µM with in a final DMSO concentrations of 0.005%, 0.68%, and 1.3% respectively. The cells were then cultured for a week with media changes on day 3 and 5. These drug concentrations were based on previous research in PGP induction with the same drugs and through email correspondence with Dr. Christopher Farrell (7, 8). Treated cells then were harvested and assessed for cell viability using the PrestoBlue assay. The highest concentration which maintained cellular viability for each drug was selected to continue on with the induction study. Cellular viability was based on the visual health of the populations as determined by cell confluence and number of dead cells counted in the media. Results from PrestoBlue experiment was analyzed using an Infinite M200 Pro-Tecan plate reader at the University of the Incarnate Word.

PGP Induction

Based on results from the dose response curve the 75µM fexofenadine and 2nM vinblastine concentrations were chosen for the PGP induction studies. The DMSO vehicle controls were prepared at a concentration of 0.68%, this matches the 75µM fexofenadine exposure as this is highest DMSO content of all treatments. The induction study was repeated three times with each exposing SKOV-3 cells to drug conditions for 42 days. Within each study

the drug exposures were performed in triplicate. The intent was to have media changeout occurring every three days and RNA isolation every seven days. Because of differences in cell growth rate due to drug exposure however some timepoints were moved to allow for additional days of growth. Throughout all 3 studies untreated control cells were maintained with RNA isolation taken at the beginning and end of each induction study.

qRTPCR

After each induction study had concluded the isolated RNA collected from the cells was analyzed for signs of PGP induction utilizing qRT-PCR. The isolated mRNA was used to synthesize cDNA using the Superscript II Reverse Transcriptase kit from Invitrogen. Expression of *ABCB1* was evaluated with customized Taqman Gene Expression Assays purchased from Thermofisher Scientific for *ABCB1* (Hs00184500_m1) and the housekeeping gene *HPRT1* (Hs01003267_m1). Expression was detected using fluorescent probes for *ABCB1* and *HPRT1* with a Bio-Rad C1000 Touch Thermocycler. The CT values provided by the thermocycler were then used to analyze the *ABCB1* gene expression relative to the expression of the housekeeping gene *HPRT1*, this is known as the delta cycle threshold (Δ CT) value. The Δ CT values for each exposure was additionally compared to the values from the untreated controls.

$$\text{Delta Ct} = \text{Ct gene test} - \text{Ct endogenous control}$$

Paclitaxel Resistance

After each induction study concluded the treated SKOV-3 cells were exposed to paclitaxel to assess if PGP induction could confer resistance to the chemotherapeutic drug. Paclitaxel was solubilized in DMSO and then diluted into water. The paclitaxel concentrations for these experiments were 0.1 μ M and 1 μ M with in a final DMSO concentrations of 0.0016% and 0.0169% respectively. SKOV-3 cells were plated onto a 96-well plate and exposed to RPMI

media with either 0.1 μ M and 1 μ M paclitaxel for 24 hours. After this period the cells were then tested for cell viability using the PrestoBlue Cell Viability Assay. Results from PrestoBlue experiment was analyzed using an Infinite M200 Pro-Tecan plate reader at the University of the Incarnate Word. This experiment was only performed at the conclusion of the second and third PGP induction studies.

Data Analysis and Statistics

The Δ CT values produced by the PCR machine were used to examine PGP expression for each study. At each timepoint, results from every exposure group was analyzed using a Student's t-test as compared to the untreated controls collected at the start of each study. Statistical analysis was performed in SPSS version 24 and Microsoft Excel 2016. Cell viability data gathered using the PrestoBlue assay after paclitaxel exposure was evaluated by comparing results to the untreated control cells.

Results

ABCB1 Expression in Treated SKOV-3 Cells

The expression level of *ABCB1* was investigated to determine if treatment with 75 μ M fexofenadine or 2nM vinblastine could induce *ABCB1* expression in SKOV-3 cells. The Δ Ct values normalizes the expression of *ABCB1* to that of the housekeeping gene *HPRT1*. Significance for each Δ Ct value was determined through comparison against the day 0 untreated controls. All of the 0.68% DMSO vehicle control samples exhibited a statistically significant decrease in *ABCB1* expression with samples collected from day two and four showing the sharpest decline ($p > 0.001$) (Figure 1). The remainder of the DMSO samples displayed a statistically significant decrease of ($p > 0.05$) in *ABCB1* expression. Samples treated with 75 μ M fexofenadine were observed to have a statistically significant decrease in *ABCB1* expression on

day 14 ($p > 0.001$) and day 31 ($p > 0.05$). None of the fexofenadine treated samples exhibited any significant signs of *ABCB1* induction (Figure 1). The 2nM vinblastine treated cells displayed no significant increase in *ABCB1* expression throughout the study (Figure 1).

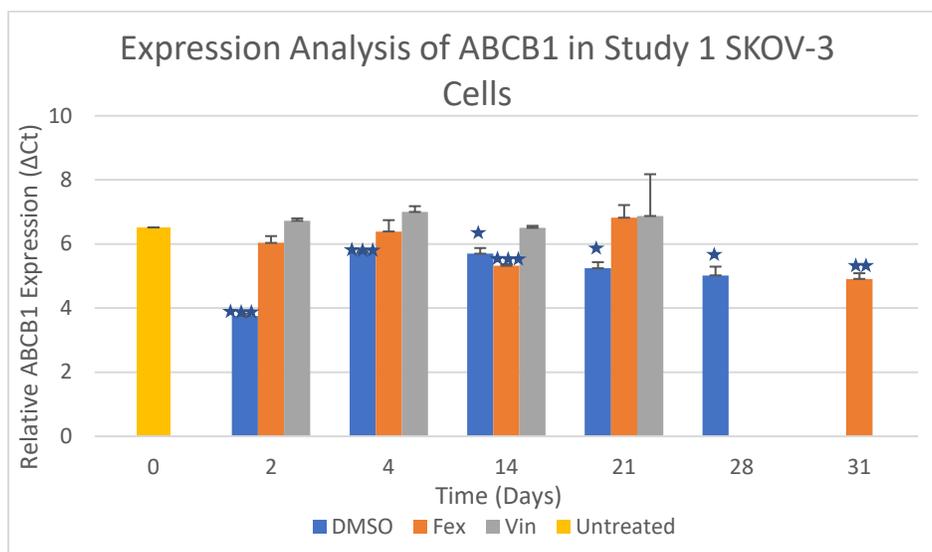


Figure 1. Expression analysis of *ABCB1* in drug treated SKOV-3 cells from the first induction study as measured by qRT-PCR. Chart utilizes ΔC_t values which compare *ABCB1* expression to that of the endogenous control gene *HPRT1*. Values were compared with the day 0 untreated cells to determine significance and were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The second induction study was carried out using the same treatments as previously described with the addition of untreated cells which ran concurrently to the other treatments and had RNA isolated on day zero and day 56. The cells from the DMSO vehicle controls were observed to have a significantly significant decrease ($p > 0.05$) in PGP expression starting from day 15 until the end of the test period (Figure 2). The 75 μ M fexofenadine treated samples from day four exhibited statistically significant decrease ($p > 0.05$) in PGP expression. All remaining samples treated with fexofenadine did not indicate any significant signs of *ABCB1* induction (Figure 2). The 2nM vinblastine treated cells displayed a statistically significant decrease ($p > 0.05$) in *ABCB1* expression in samples starting from day 15 until the end of the test period

(Figure 2). The vinblastine treated cells displayed *ABCB1* expression levels beneath that of the other treatments for the duration of the study.

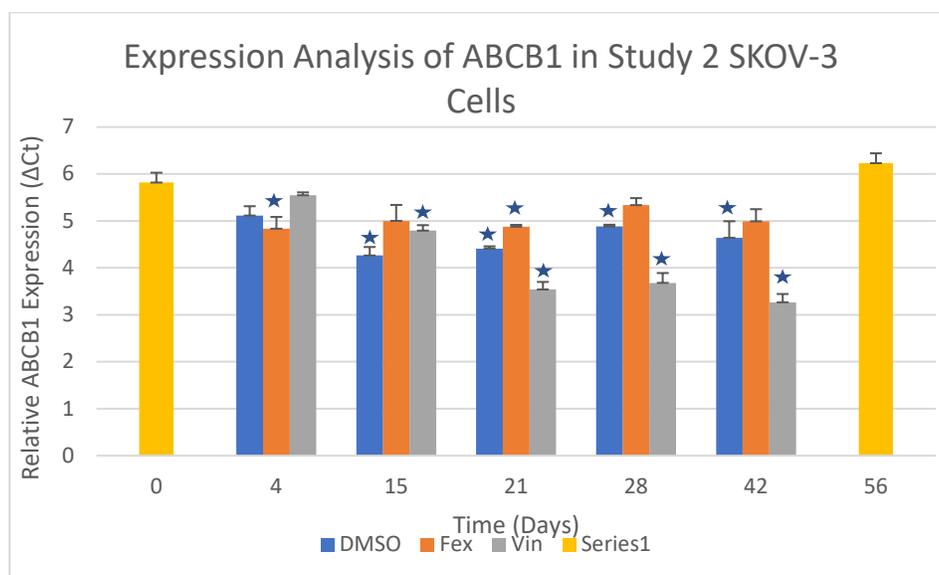


Figure 2. Expression analysis of ABCB1 in drug treated SKOV-3 cells from the second induction study as measured by qRT-PCR. Chart utilizes ΔC_t values which compare ABCB1 expression to that of the endogenous control gene HPRT1. Values were compared with the day 0 untreated cells to determine significance and were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The third induction study utilized the same drug treatments as the previous studies and maintained a population of untreated control cells which had RNA isolated on day zero and 42. On day 42 of the study the DMSO vehicle samples displayed a statistically significant decrease ($p > 0.01$) in *ABCB1* expression (Figure 3). The 75 μ M fexofenadine treated cells were observed to statistically significantly decrease ($p > 0.01$) in samples collected from day 21 (Figure 3). Additionally, fexofenadine treated samples from day 42 displayed a statistically significant decrease ($p > 0.001$) in ABCB1 expression. Both the DMSO vehicle and 75 μ M fexofenadine treated cells overall maintained a steady decrease in ABCB1 expression throughout the study. The 2nM vinblastine treated cells exhibited a statistically significant increase ($p > 0.05$) in

ABCB1 expression on samples from day 14. Vinblastine treated cells also displayed a significant decrease ($p > 0.05$) in *ABCB1* on day 42 (Figure 3).

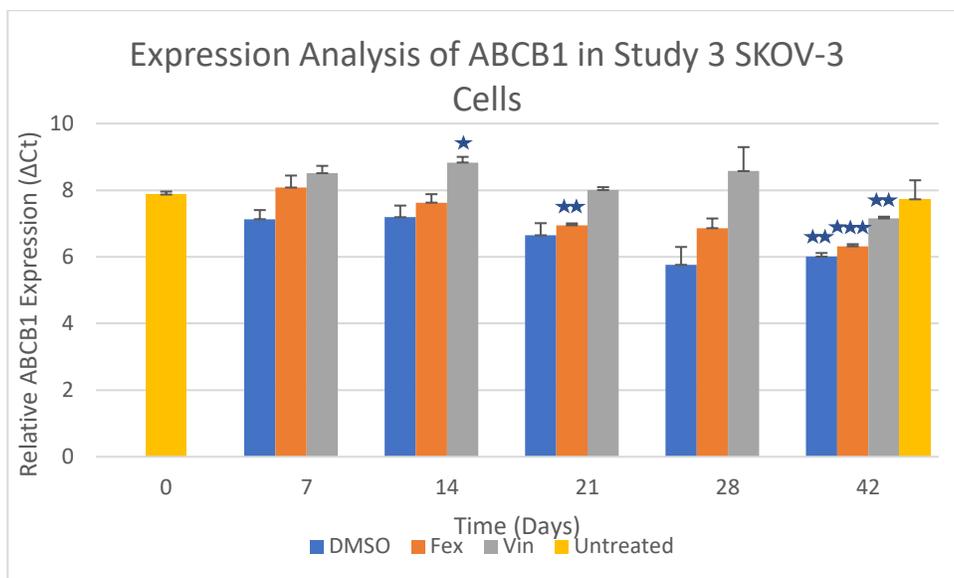


Figure 3. Expression analysis of ABCB1 in drug treated SKOV-3 cells from the third induction study as measured by qRT-PCR. Chart utilizes ΔC_t values which compare ABCB1 expression to that of the endogenous control gene HPRT1. Values were compared with the day 0 untreated cells to determine significance and were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Resistance to Paclitaxel in Treated SKOV-3 Cells as Determined by PrestoBlue Viability Assay

In order to assess if PGP induction could result in the development of drug resistance the treated SKOV-3 cells were then exposed to paclitaxel. After the SKOV-3 cells from the second induction study were treated for 42 days with each drug condition, they were removed from the fexofenadine or vinblastine exposure and then exposed one of two different concentrations of paclitaxel and tested for cell viability on Day 51. The charts below reflect the absorbance of PrestoBlue as compared to media only controls, groups with higher absorbance indicate superior survival against paclitaxel exposure. Based on the recommendation of the protocol provided by Thermofisher, cell viability was detected after 20 minutes of incubation. For the 0.1 μ M paclitaxel exposure experiment, the DMSO control and 2nM vinblastine treated cells overall

exhibited similar survival when compared to the untreated controls. The 75 μ M fexofenadine cells had the worst survival with absorbance nearly half of that of untreated controls (Figure 4a). For the 1 μ M paclitaxel exposure experiment, all groups exhibited lower survival than the previous 0.1 μ M exposure. The 2nM vinblastine treated samples showed the highest survival of all treatments (Figure 4b). The untreated control cells had the second-best survival falling just behind the vinblastine cells. Both the DMSO control and 75 μ M fexofenadine treated cells both displayed poor survivability when compared the vinblastine cells exposed to the same paclitaxel treatment (Figure 4b).

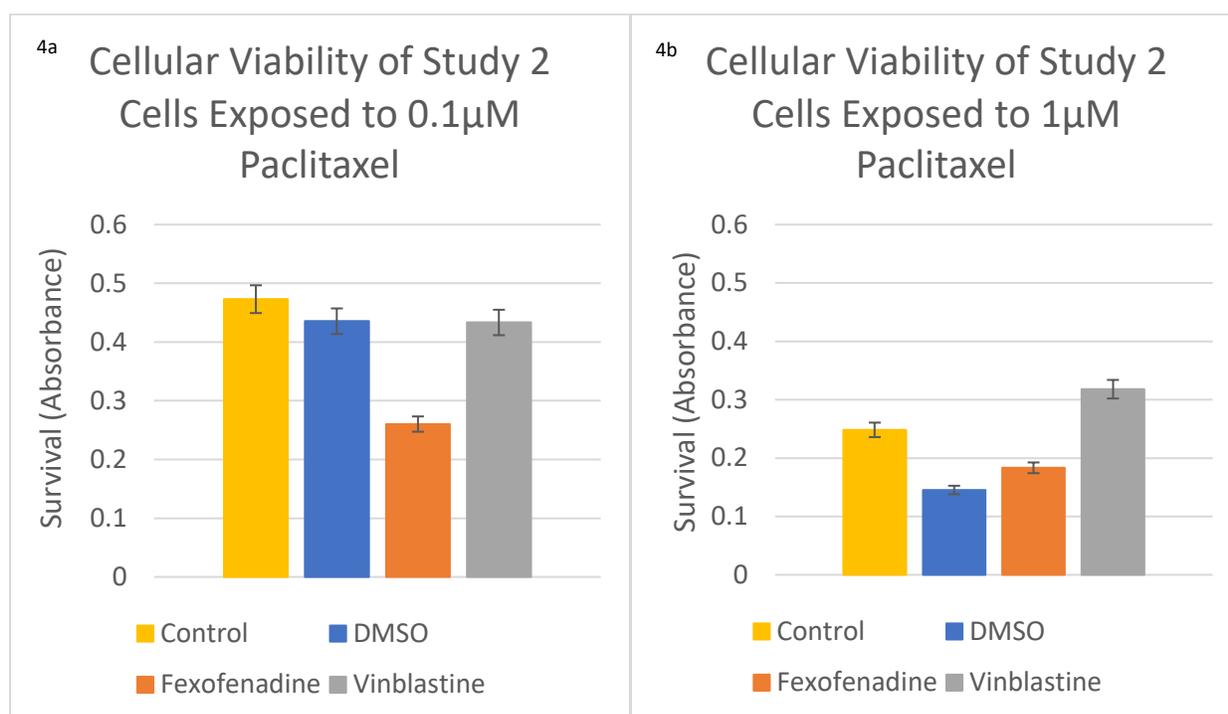


Figure 4. Cell viability analysis of treated SKOV-3 cells after exposure to paclitaxel to ascertain presence of resistance to chemotherapeutic treatment. SKOV-3 cells from the second induction study (Day 51) were exposed to 2 different concentrations of paclitaxel and measured for cell viability after 20 minutes using PrestoBlue assay. The media corrected absorbance values were read at 570 nm and normalized to the 600 nm values. Cells exhibiting resistance to paclitaxel should have greater cell viability. (a) Treated SKOV-3 cells exposed to 1 μ M of Paclitaxel. (b) Treated SKOV-3 cells exposed to 0.1 μ M of Paclitaxel.

After the third induction study had concluded the cells were exposed to 0.1 μ M of paclitaxel the DMSO treated samples exhibited the greatest survival of all treatments. The untreated control and 75 μ M fexofenadine treated cells exposed to paclitaxel exhibited very similar survival with the untreated controls showing slightly greater absorbance. The 2nM vinblastine treated cells fared the worst with survival lower than any of the other treatments (Figure 5b). SKOV-3 cells exposed to 1 μ M of paclitaxel displayed similar trends to those found in the 0.1 μ M paclitaxel exposure. The greatest overall survival took place in the DMSO control samples (Figure 5b). The fexofenadine treated cell had very similar survival to the DMSO controls while the untreated control cells fared slightly worse. Cells treated with 2nM vinblastine had the worst survival with an absorbance less than half of any other treatment exposed to the same Paclitaxel dose (Figure 5b).

Discussion

This study aimed to investigate the expression of *ABCB1* in SKOV-3 ovarian cancer cells after treatment with vinblastine and fexofenadine, drugs identified as PGP substrates. Vinblastine is a powerful chemotherapeutic drug which has a documented history as a PGP inducer, and thus, it was selected as a positive control for this study (13, 16). Fexofenadine is an over-the-counter antihistamine commonly used to relieve allergy symptoms that is also a known PGP substrate (16). Fexofenadine was chosen for this study because of the implications this drug could have for patients before a cancer diagnosis. If treatment with fexofenadine can alter *ABCB1* expression and its associated regulatory mechanisms in cancer cells it could impact the efficacy of chemotherapeutic drugs like paclitaxel. PGP induction specifically has been

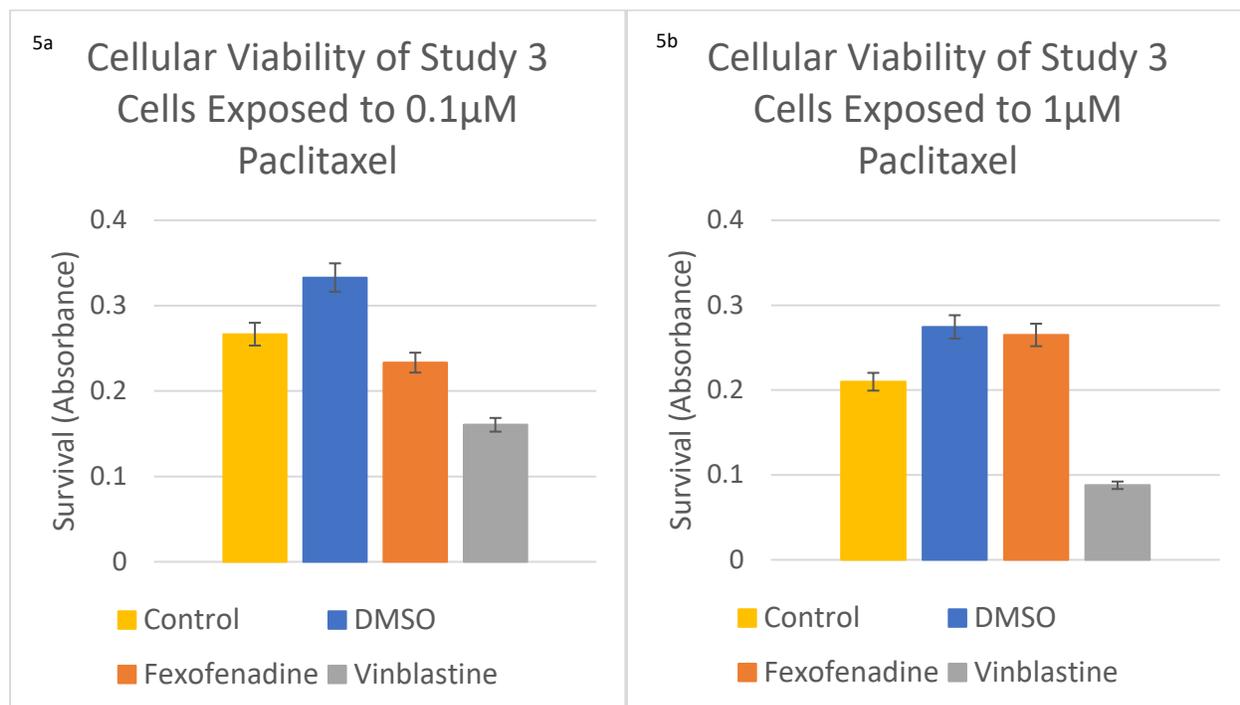


Figure 5. Cell viability analysis of treated SKOV-3 cells after exposure to paclitaxel to ascertain presence of resistance to chemotherapeutic treatment. SKOV-3 cells from the third induction study (Day 51) were exposed to 2 different concentrations of paclitaxel and measured for cell viability after 20 minutes using PrestoBlue assay. The media corrected absorbance values were read at 570 nm and normalized to the 600 nm values. Cells exhibiting resistance to paclitaxel should have greater cell viability. (a) Treated SKOV-3 cells exposed to 1 μ M of Paclitaxel. (b) Treated SKOV-3 cells exposed to 0.1 μ M of paclitaxel.

identified as the target of an ever-growing number of inquiries involving the development of drug resistance. While the resulting alterations in gene expression through drug exposure could be transient in nature when produced in a lab environment, the acquisition of drug resistance through ABC transporter expression remains a relevant concern with the clinical community (23, 24).

Across all three PGP induction studies performed in this experiment, both the fexofenadine and DMSO vehicle controls consistently did not induce significant *ABCB1* induction. Results from the second and the third studies further indicate that both the

fexofenadine and DMSO controls display *ABCB1* levels beneath that of untreated cells. The consistently similar results displayed by these two groups could be due to the fact that both exposures had the same 0.068% DMSO content. The vehicle controls were calculated to have the same percentage of DMSO as was used to make the 75 μ M fexofenadine exposure. Despite the similarity between these results, the DMSO vehicle cells in all three induction studies consistently exhibited more change with an overall negative trend as each study proceeded. Although the final DMSO concentration in these samples was beneath the recommended 0.1%, it is possible that the DMSO content was still enough to cause the PGP expression in SKOV-3 cells to fall. As this same amount of DMSO was used to solubilize fexofenadine in this study, this could indicate that the potential induction effects of fexofenadine exposure could have been counteracted by the toxicity of the DMSO. This conclusion however would require further investigation to verify.

The vinblastine treated cells from the first and third induction studies were the only exposures to demonstrate an increase in *ABCB1* expression. In the case of the first study however the values were not significant. The only vinblastine treated cells which did exhibit a significant increase in *ABCB1* expression was from the third induction on day 15. Subsequent samples from the third induction study actually began to show a decrease with *ABCB1* expression dipping beneath the untreated controls by the final day of the experiment. This could be a result of the transient nature of drug-induced *ABCB1* induction documented in a study by Miklos et al which utilized SW480 cells. In a study published in the Journal of the National Cancer Institute, researchers further designated vinblastine specifically as a chemotherapeutic additionally capable of inducing stable *ABCB1* expression in K562 cells, but not within HL-60 leukemia cells (24). The diversity of results vinblastine exposure can have among different cell types could

contribute to answering why exactly vinblastine failed to act as a reliable positive control in this study.

The results from the vinblastine treated cells in second induction study were very different than the other two studies as they displayed a notable decrease in *ABCBI* expression which did not level out until the third week of the study on Day 21. Like the third study all drug treated cells had lower *ABCBI* expression when compared to the untreated SKOV-3 cells. This downward trend in the early days of the study may be related to an incident which occurred on the 10th day of the study during which the incubator failed and was no longer supplying CO². During the transfer to a functioning incubator the cells were outside of a controlled environment for more than five hours. When this occurred the first and second induction studies were both running concurrently so the incubator had flasks with cells from both studies. The reason however that one does not see the possible implications of this incident in the first study is because the second study was started two weeks after the first. By day 10, the cells from study two had only experienced three days of growth since their last passage. The cells from the first study on the other hand were on day 27 and had already experienced a full week of growth since their last passage. To compensate for this the cells from both studies were given extra time to grow in an incubator at a separate facility as both were unable to reach confluence on schedule. Although this may have had an adverse effect on the cellular growth rate and morphology it cannot be fully concluded that the downward expression of *ABCBI* displayed was directly the caused by the incubator malfunction.

After generating a best fit trendline for all of the ΔC_t values with each condition for all three studies, the slopes of each line were analyzed to see the overall trend of how each treatment affected the cells. Trendlines for DMSO and fexofenadine treated cells in all three studies were

negative, meaning that the drugs overall decreased the expression of *ABCB1* in SKOV-3 cells. While extremely similar, these negative trends do not deviate far from *ABCB1* expression in untreated cells on shown on day zero (Figure 6a, 6b, 6c). This indicates that DMSO and fexofenadine treatments did not induce significant change in our SKOV-3 cells after 42 days of exposure. Trendlines generated for the vinblastine treated cells exhibit a notable degree of difference from study to study with only the first study having a positive trend towards *ABCB1* induction (Figure 6a). Similar to the first study, the trendline for the vinblastine treated cells in the third study also stays above that of the DMSO control and fexofenadine cells (Figure 6c). The trendline generated from the second study however exhibits a noticeable negative slope when compared to both the other treatments in the same study and the other vinblastine treatments from the other studies (Figure 6b). This statistically significant ($p < 0.05$) decline in *ABCB1* expression requires further exploration to properly explain.

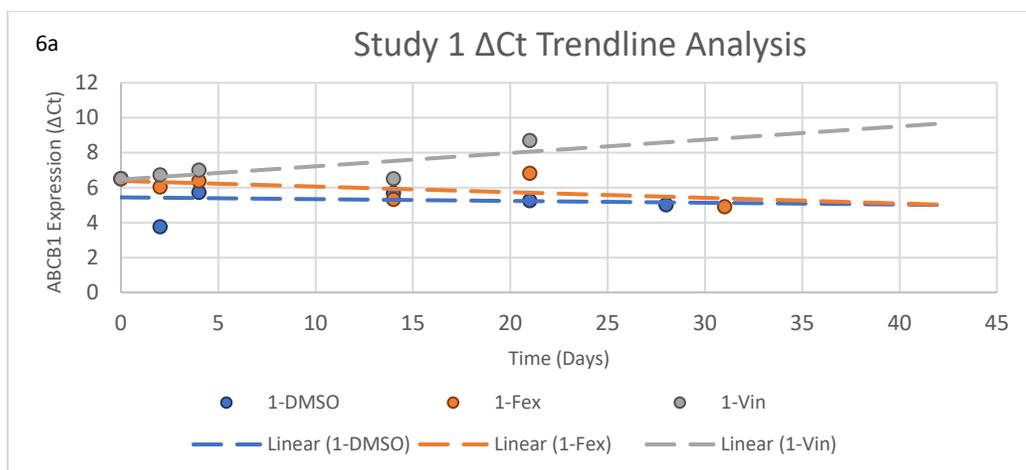


Figure 6a. Trendline expression analysis of *ABCB1* in drug treated SKOV-3 cells from the first induction study as measured by qRT-PCR. Utilizes ΔC_t values to generate best fit trendlines to visualize overall effects of each treatment on *ABCB1* expression.

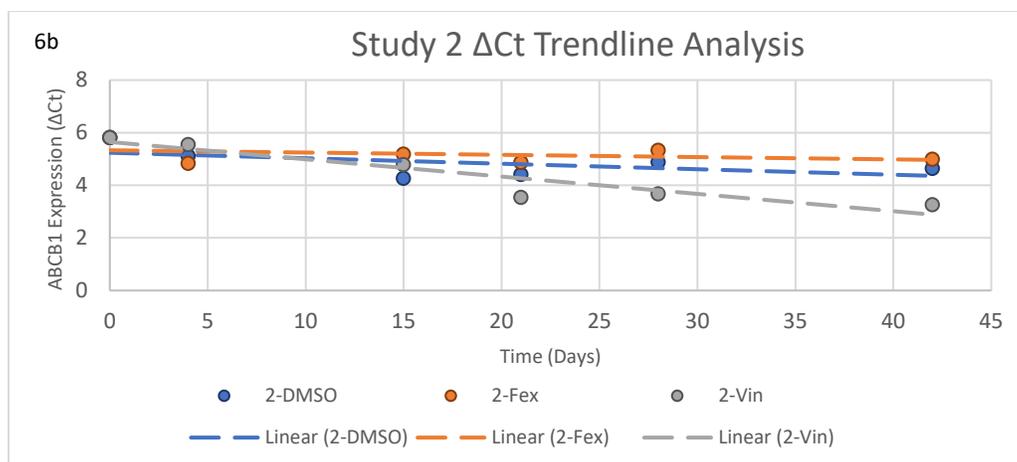


Figure 6b. Trendline expression analysis of ABCB1 in drug treated SKOV-3 cells from the second induction study as measured by qRT-PCR. Utilizes Δ Ct values to generate best fit trendlines to visualize overall effects of each treatment on ABCB1 expression.

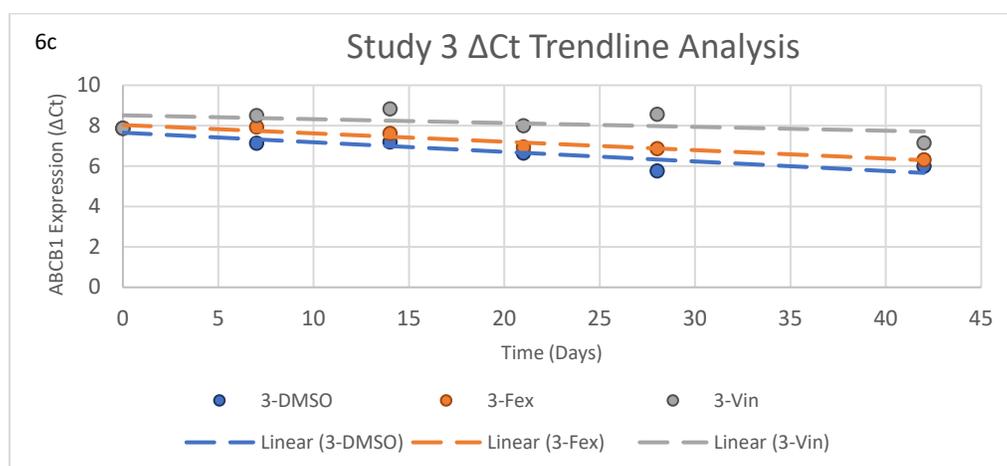


Figure 6c. Trendline expression analysis of ABCB1 in drug treated SKOV-3 cells from the third induction study as measured by qRT-PCR. Utilizes Δ Ct values to generate best fit trendlines to visualize overall effects of each treatment on ABCB1 expression.

In order to statistically examine the reproducibility of PCR results across the three induction studies, the confidence intervals were identified for the slopes of each treatment's best fit trendline across all of the induction studies. Using a linear regression model, Appendix A shows the results of this analysis which was conducted in IBM SPSS (Statistical Package for the Social Sciences). If the slope of each trendline all fit into the same 95% confidence intervals,

then there is insufficient evidence to conclude that the slope of each line differs significantly. The slope lines for fexofenadine treated cells from study one and three do not differ significantly; thus, there is insufficient evidence to say that the results of these treatments differ in SKOV-3 cells. The slope line of the fexofenadine cells from the second induction study does somewhat overlap with that of the first, but not of the third study. This indicates that the cells between the second and third study do show significantly different results from their respective treatments. The slopes generated for DMSO-treated cells all overlap meaning that there is insufficient evidence to conclude that results of this exposure differed between the three studies for this treatment. Finally, the slopes generated from the trendlines in vinblastine treated cells overlapped in cells from study one and three. This indicates that the change in *ABCB1* expression did not differ in a significant way between cells of those studies. The slope for vinblastine treated cells in study two, however, does differ significantly from the similarly exposed cells from either the first or third studies. This variance present in the results SKOV-3 cells indicates the need for further testing as to whether either substrate could reliably induce PGP in SKOV-3 cells.

While the data was unable reliably demonstrate fexofenadine or vinblastine induced *ABCB1* expression in our SKOV-3 cells, the idea of PGP substrate drugs possibly acting as inducers remains a query worth exploring. Across numerous cell types, the expression of *ABCB1* has been shown to play a role in the absorption and secretion of numerous anticancer drugs (10). The anticancer drug vinblastine's association with *ABCB1* expression especially seems noteworthy based on findings of studies which utilize other cell types. For example, a study performed by Harmsen et al. in 2010 utilized a LS180 colon cancer cell line to verify that anticancer drugs, such as paclitaxel and vinblastine could induce PXR-mediated PGP overexpression. (9). Another study which utilized human neural stem/precursor cells (hNSPCs)

found that treatment with vinblastine resulted in the drug-induced expression of *ABCB1* (13). Using a SW840 colon cancer cell line with known *ABCB1* overexpression, Miklos et al. attempted to verify the impact *ABCB1* expression could have on resistance to the chemotherapeutic triapine, which itself is a substrate of PGP. While they concluded that triapine was only a weak substrate for PGP, they did interestingly find that treatment with triapine resulted in the upregulation of *ABCB1* in their SW840 colon cancer cells. They concluded that this upregulation was due to the stress the cells underwent with exposure to triapine (23). Whether or not SKOV-3 cells could exhibit a comparable mechanism that could alter *ABCB1* expression when under similar pressure may be a topic worth further investigation.

In addition to any *ABCB1* gene expression effects, some of the drug treatments utilized in this study did have an observable effect on the morphology and health of the SKOV-3 cells. The normal untreated SKOV-3 cells, as well as those treated with 0.068% DMSO, had a ‘spiderlike’ structure that when plated with two million cells would reach confluence inside of a T-75 flask after seven days (Figure 7a, 7b). Using the official ATCC documentation on the cells as well as the untreated controls as a baseline for average SKOV-3 growth patterns, the 75 μ M fexofenadine treated cells appeared to display somewhat augmented growth. A T-75 flask of untreated cells generally was expected to be confluent within a week but fexofenadine treated cells often would reach confluence in as few as five days despite being initially plated with the same number of cells. While these flasks would of course yield much higher cell counts than the other treatments the morphology of the SKOV-3 cells would remain largely unchanged (Figure 7c). The 2nM vinblastine treated cells developed an irregular globular shape that would form clusters or islands of growth rather than spread out across the plate (Figure 7d). In addition to these new characteristics, the vinblastine treated cells often were in poor health and low confluence after

each week, sometimes requiring a higher plating density of cells to even make the required numbers of cells for each week of the experiment. While this obviously is tied to the potency of vinblastine as a chemotherapeutic agent the effect this more extreme environment could have resulted in innumerable changes on the gene expression of the SKOV-3 cells. The exact relation between the health of the cells, as indicated by higher cell counts or changes in their morphology, and the expression of *ABCB1* remains unknown and requires further investigation.

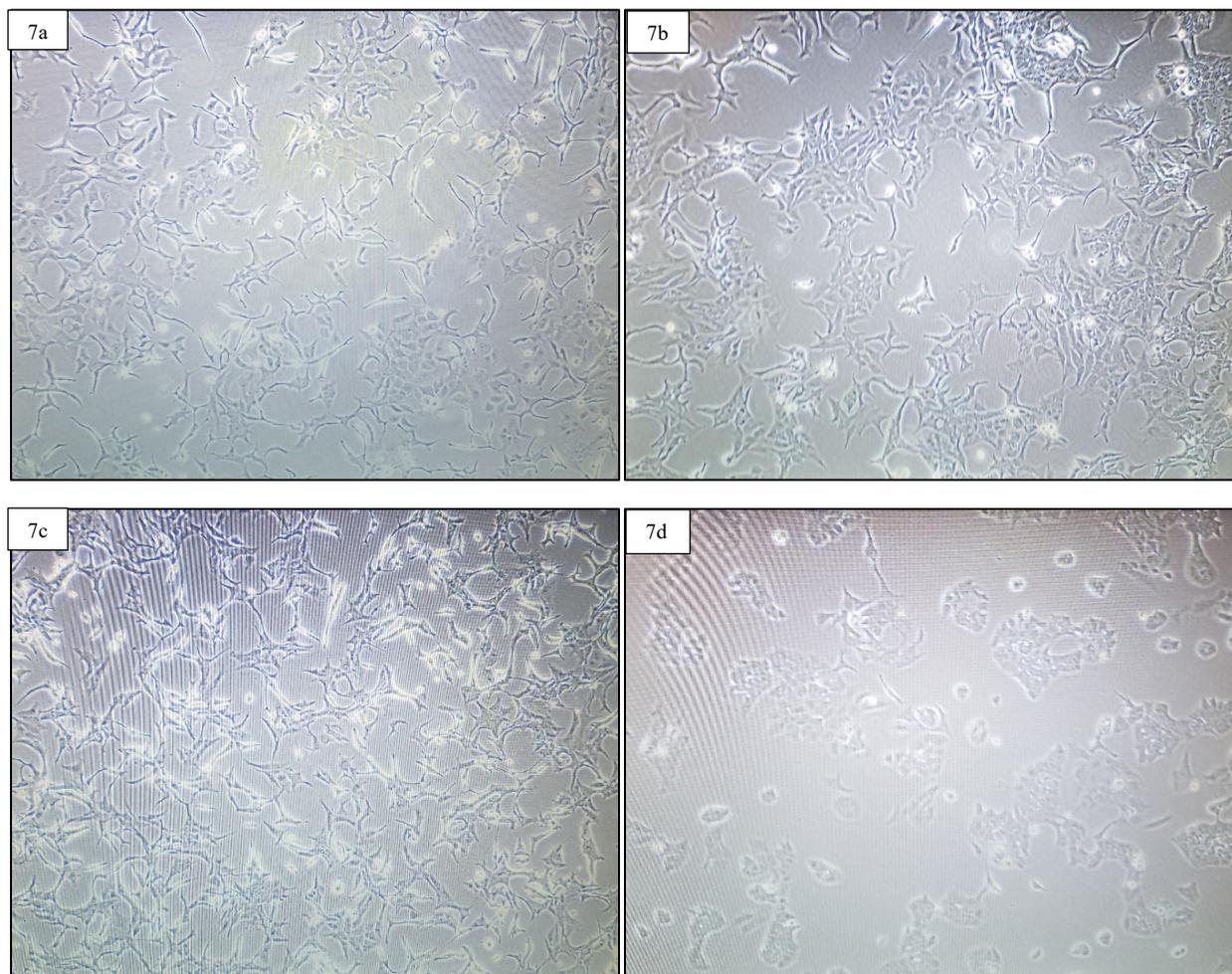


Figure 7. SKOV-3 cells imaged under each under each treatment after 5 days of growth within a CO₂ incubator. (a) Untreated SKOV-3 cells. (b) 0.068% DMSO treated SKOV-3 cells. (c) 75µM fexofenadine treated SKOV-3 cells. (d) 2nM vinblastine treated SKOV-3 cells.

The second aim of this experiment was to identify if SKOV-3 cells with augmented *ABCB1* expression would exhibit resistance to treatment with the chemotherapeutic drug paclitaxel, which itself is a PGP substrate. In the cells taken from the second induction study, there was a notable difference in the survivability between the cells of each paclitaxel exposure, with the primary commonality being that the fexofenadine treated cells proved most susceptible to the cytotoxic effects of the drug under both conditions. In the cells from the second study exposed to 1 μ M paclitaxel, the greatest survivability was found in vinblastine treated cells. The next greatest survivability to 1 μ M paclitaxel was in the untreated control cells followed by the fexofenadine and lastly the DMSO treated cells. The cells from the second study exposed to 0.1 μ M displayed markedly different results from the 1 μ M cells with the untreated control cells showing the strongest survivability. The DMSO and vinblastine treated cells trail just behind the untreated cells with notable survival towards paclitaxel while the fexofenadine treated cells again fared the worst. Based on the expression analysis from these cells the DMSO and fexofenadine treated cells showed no significant signs of *ABCB1* induction so it unlikely that their susceptibility to paclitaxel treatment was related to that factor. Furthermore, the untreated baseline cells in both experiments exhibited both higher levels of *ABCB1* than all other treatments. While noteworthy these trends are hardly consistent as the *ABCB1* expression in baseline cells is not significantly induced. The *ABCB1* expression in the vinblastine treated cells, which fared best against the 1 μ M paclitaxel exposure, additionally displayed greatly reduced gene expression which taken altogether indicates the need for further testing.

In cells taken from the third induction study, the untreated, DMSO, and fexofenadine exposed cells all exhibited similar survivability for both concentrations of paclitaxel. In all of these samples, the cells exposed to the higher 1 μ M dose resulted in lower survivability across all

treatments than the cells exposed to the lower 0.1 μ M dose. The greatest survivability was displayed in DMSO samples, but since these cells showed very little difference in *ABCB1* expression to either the untreated controls, or the fexofenadine treated cells, the exact reason for this is unknown. By large margin the weakest survivability was found in vinblastine treated cells for both paclitaxel concentrations which noticeably varied from all other treatment types. One possible reason for this greatly diminished survivability may be due to the low yield and poor health of the vinblastine treated cells on day 49 of their treatment before they were exposed to paclitaxel. The diminished survival of the vinblastine treated cells towards paclitaxel treatment could be linked to the high toxicity of vinblastine which already had done considerable damage to the SKOV-3 population. Additionally, the visible health of the untreated, DMSO, and fexofenadine treated cells were all relatively at the same. Whether the relative health of the cells prior to exposure to paclitaxel could be the cause of this variance, however, will require further investigation to verify as the *ABCB1* levels of the cells in each treatment by that time cannot be verified and thus true causality cannot be assumed.

Altogether, the data from our paclitaxel exposures appears to illustrate an association between SKOV-3 cells with reduced *ABCB1* expression and a degree of paclitaxel resistance as augmented survivability would indicate reduced sensitivity to the drug's cytotoxic effects. This stands in opposition to established and well-described research which indicates that induction of *ABCB1* expression is a viable mechanism for development of resistance to drugs such as paclitaxel (9, 21, 22). While there are various differing factors which contribute the differences found in these previous studies, they all contribute knowledge which may help explain the variance found in our own results. For example, a study performed by Medical University of Vienna utilized a LS180 colon cancer cell line to verify that anticancer drugs such as paclitaxel

and vinblastine are shown to induce a PXR-mediated PGP overexpression. Furthermore, it has been shown that PXR activation is associated with the metabolic reduction of cytotoxic agents, such as doxorubicin in colon cancer cells (9). This particular study identifies a unique method by which resistance to doxorubicin is conferred through PXR, while results from additional studies also suggest that *BCBI* activation is a fundamental mechanism for acquired drug resistance to taxanes, such as paclitaxel in ovarian cancer cells (22). It has also been found that paclitaxel resistance in SKOV-3 cells is not achieved through the usual PXR-mediated method but rather through a novel caspase-3 and caspase-9 independent pathway (20). While not a definitive answer, it does set a precedent that paclitaxel resistance can be achieved in numerous cell types through methods which have the potential to be unique to each cell type being studied.

In conclusion, the experiments carried out with this ovarian cancer cell line did not confirm the hypothesis that SKOV-3 cells treated with PGP substrates fexofenadine and vinblastine could induce *ABCBI* gene expression. If further testing were to occur on this project, then the addition of cells with confirmed PGP induction would be added for a strong positive control for both aims. Additionally, running a drug dosage curve at the start of the project with higher concentrations may help ensure that any changes seen in *ABCBI* expression within the cells are actually due to drug exposure rather than other external factors. Finally, it could be beneficial to run qRT-PCR at multiple points throughout the study, rather than all at once at the end. This would better qualify the results of the paclitaxel exposure experiment as the test for resistance would avoid the possible long-term effects of the drugs and for sure utilize cells which exhibit verified *ABCBI* induction.

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Appendix: Linear Regression Coefficients Analysis

Fexofenadine Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	6.385	.418		15.266	.000	5.224	7.546
	Time	-.032	.025	-.535	-1.267	.274	-.103	.038

a. Dependent Variable: Fex Study 1

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	5.331	.255		20.893	.000	4.623	6.040
	Time	-.009	.011	-.366	-.787	.475	-.039	.022

a. Dependent Variable: Fex Study 2

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	8.034	.129		62.296	.000	7.676	8.392
	Time	-.041	.006	-.966	-7.471	.002	-.057	-.026

a. Dependent Variable: Fex Study 3

DMSO Control Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	5.444	.625		8.705	.001	3.708	7.180
	Time	-.010	.040	-.126	-.254	.812	-.122	.102

a. Dependent Variable: DMSO Study 1

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	5.238	.342		15.300	.000	4.287	6.188
	Time	-.021	.015	-.578	-1.417	.230	-.062	.020

a. Dependent Variable: DMSO Study 2

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	7.652	.258		29.713	.000	6.937	8.367
	Time	-.047	.011	-.906	-4.275	.013	-.078	-.017

a. Dependent Variable: DMSO Study 3

Vinblastine Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	6.462	.454		14.219	.001	5.015	7.908
	Time	.076	.040	.743	1.920	.151	-.050	.202

a. Dependent Variable: Vin Study 1

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	5.646	.291		19.429	.000	4.839	6.453
	Time	-.066	.013	-.934	-5.250	.006	-.101	-.031

a. Dependent Variable: Vin Study 2

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	8.512	.413		20.600	.000	7.365	9.660
	Time	-.019	.018	-.472	-1.071	.345	-.068	.030

a. Dependent Variable: Vin Study 3