Honors Thesis Proposal

In vitro characterization of ovarian cancer cell drug resistance to microtubule-disrupting agent FROST450, an experimental therapeutic

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Introduction

Chemotherapies are a long-established cancer treatment. Among these therapies are drugs that either inhibit or stabilize microtubule polymerization as a means of preventing cell division. Since microtubules are required for cell division and cancer cells are fast, often continuously dividing, they are particularly sensitive to these agents. Taxanes and vinca alkaloids are the main drugs in this class, and significant treatment success has been achieved with these agents. In fact, taxanes and vinca alkaloids are the most successful cancer treatments developed to date, having dramatically altered survival across a number of cancer types.

Despite this, tumors in patients treated with these drugs are likely to develop resistance to these treatments, a result of selection for mutations that drive drug resistance. Several mechanisms of taxane and vinca resistance have been identified, though others remain poorly characterized. These mechanisms vary in how much cross-resistance is generated against other microtubule targeting or chemotherapeutic agents. One broad mechanism of resistance is through general or 'multi-drug' resistance. The molecular mechanism of this form of resistance is the action of ABC transporters that serve to export drug-like molecules from the cell, reducing the intracellular concentration and allowing the cell to survive high drug concentrations. The main transporter of this type, P-glycoprotein (PGP), is encoded by the multi-drug resistance 1 gene (MDR1). Taxanes and vincas are substrates of PGP, and cells that increase the expression of this transporter become resistant to these treatments. Interestingly, PGP upregulation can be selected for during treatment with other drugs that are also PGP substrates, meaning pre-treatment with any PGP substrate drug can drive resistance to all other treatments that are PGP substrates. Taxane (or vinca) resistance may also occur through non-PGP mechanisms, some of which may or may not provide cross resistance to other microtubule targeting agents. One example is increased expression of βIII tubulin, which confers resistance to a number of microtubule targeting agents, but not to other chemotherapeutics. The most selective mechanism of resistance appears to be point mutations in tubulin genes, which are likely to confer resistance to only specific classes of microtubule targeting agents, or even only to specific analogs within a class. No matter the mechanism acquired, taxanes and vincas can be rendered either partially or completely ineffective in the clinic and a second-line anticancer treatment that overcomes those acquired resistance mechanisms is required. These mechanisms will either be acquired through new genetic mutation or from selection of a small subset of the cell population that already has the resistant genes.

Acquired taxane resistance occurs frequently in a number of caner types, but is particularly problematic in ovarian cancer. Standard first-line treatment for ovarian cancer is normally a combination of a DNA-damaging platinum compound (i.e. cisplatin or carboplatin) and a

microtubule-stabilizing taxane compound (i.e. paclitaxel). Taxane resistance almost inevitably develops and the primary treatment strategy will begin to fail. Several second-line treatment options exist, but these second-line drugs tend to be highly focused on specific ovarian cancer subtypes and have limited success in generating positive patient responses. And, current second-line treatments typically generate poorer patient responses than the first line therapy.

In this study, we propose to work with a novel microtubule stabilizer, FROST450. FROST450 has proven potential for ovarian cancer; it arrests cell growth of a number of ovarian cancer cell lines and has proven tumor growth inhibition activity in animal models, including ovarian cancer xenografts. Also in previous studies, breast cancer cells with acquired resistance to paclitaxel demonstrate no increased resistance to FROST450; there is no taxane-FROST450 cross-resistance in cell lines that have acquired taxane resistance. Furthermore, patient-derived xenografts that are refractory to taxane treatment remain susceptible to FROST450, including examples where total tumor ablation are observed. This indicates FROST450 can remain effective as a treatment even after taxane resistance emerges in the clinic.

Our hypothesis, therefore, is that the reverse will be true: ovarian cancer cells with acquired resistance to FROST450 will demonstrate no acquired resistance to taxanes. We also hypothesize that cells resistant to FROST450 will display genetic and morphological changes different from established mechanisms of taxane resistance. To test these hypotheses, we have three specific aims:

SPECIFIC AIM 1: Identify genetic changes associated with acquired resistance to FROST450.

SPECIFIC AIM 2: Identify cellular changes associated with acquired resistance to FROST450.

SPECIFIC AIM 3: Determine the resistance of FROST450-resistant ovarian cancer cells to other antimitotic agents

Our long-term goal is to continue validating the potential of FROST450 as a treatment for ovarian cancer and prepare it for clinical trials in this disease. Identification of mechanisms of resistance will allow development of patient selection strategies in clinical testing. Patients can be screened for pharmacogenomics markers of resistance to FROST450 prior to treatment in order to select those that are likely to respond to this novel treatment. This study reveal how to position FROST450 in the clinic in relation to approved microtubule targeting agents. Can FROST450 be used in cases where taxane resistance has emerged? Do we expect a different result when treating patients with FROST450 than was achieved with ixabepilone or eribulin? Do we have reason to suspect that there will be any drug response differentiation from indibulin? If FROST450 resistance is present or emerges, would taxanes or ixabepilone be expected to be effective?

Methods

Cell culture

A2780 cells whose identity was validated prior to shipment were used for all experiments outlined here. Cells were grown in RPMI medium with 10% FBS at 37˚C and 5% CO2. In order to develop isogenic subclones resistant to FROST450, cultures were treated with FROST450 at a designated concentration. The FROST450 concentration was maintained until surviving cells exhibited normal proliferation in the presence of the compound, at which point the cells were considered resistant to that drug concentration. These newly resistant cells could then be exposed to higher dose levels in another iteration, thus generating higher and higher levels of resistance. Within several passages of achieving a resistance level, the new isogenic resistant cell line was cryogenically frozen and stored in liquid nitrogen.

Sequencing DNA and RNA to test for genetic changes

Both genomic DNA and total mRNA was extracted from parental A2780 cells and each isogenic resistant cell line subclone developed. For DNA extraction, cells were lysed with cell lysis solution and then DNA was separated out via centrifugation and precipitation with ammonium acetate. DNA was stored at 4˚C for later sequencing and bioinformatics analysis designed to identify mutation associated with the emergence of resistance, if needed in subsequent characterization of the cell lines. Total RNA was extracted using the Qiagen RNeasy kit, and then converted to cDNA by reverse transcription using the Qiagen RT-PCR kit. cDNA from each cell line, including parental A2780 cells and the isogenic resistant subclones, was sequenced using Illumina Hiseq sequencing, 200 cycles with paired ends.

Observing qualitative and quantitative morphological changes in resistant lines

Cells were grown on collagen-coated coverslips in the desired condition, then processed for fluorescence imaging. Briefly, cells were washed with ice cold Ringer's saline then fixed with 4% paraformaldehyde on ice for 20 minutes. Cells were stained with phalloidin, MitoTracker, or the appropriate antibody, then imaged by microscopy.

Measuring cross-resistance to other anticancer agents

The potency of drugs in parental and isogenic resistant A2780 cells was measured by assessing proliferation and viability at a range of doses. Each dose was tested in quadruplicate. The resulting data was plotted and an EC50 was calculated based on a sigmoidal line fit to the data. Cells were seeded into well plates, cultured for 24 hours, dosed with the desired drug(s) at the desired concentration(s), and cultured an additional 72 hours. Viable cells in each well were quantified using ATPlite reagent.

Expected Findings

SPECIFIC AIM 1: Identify genetic changes associated with acquired resistance to FROST450.

As cells acquire and increase resistance to FROST450, we expect genetic changes to occur and be reinforced. We expect that an initial change will confer beginning, low-level resistance and that reinforcement of the genetic change will allow cells to reach ever higher levels of resistance. A single genetic alteration is expected to confer some maximum limit of resistance. Once the maximum level of resistance based on that one particular mutation type is reached, cells can only achieve higher levels of resistance by acquiring an entirely new mechanism of resistance that is based on a different and new genetic change. Thus, in our efforts to develop cell lines with acquired resistance to FROST450, we expect to see a novel genetic alteration in the least resistant cell line subclone, to see this mutation somehow reinforced in subclones with slightly higher levels of resistance, and finally to see completely new genetic alterations in subclones with dramatically increased resistance. The purpose of this aim is to identify the molecular basis of resistance by characterizing genetic changes in cell lines with different levels of resistance.

A major mechanism of cancer drug resistance is by increased expression of the efflux pump MDR1. This mechanism drives broad-spectrum drug resistance that is not restricted to microtubule-targeting drugs. This efflux pump is thought to drive large increases in resistance. Since FROST450 evades MDR1, we expect that changes in MDR1 expression will not be associated with resistance, and that large gains in resistance may therefore not be achieved. In practice, this appears to be the case. MDR1 expression changes generate rapid and large gains in resistance, but we are unable to generate large gains in resistance, with minor resistance gains occurring only after extended periods of dosing. In fact, A2780 cells become resistant to another microtubule targeting agent that evades PGP, ixabepilone, in a strikingly similar way (small gains that take extended dosing periods). This suggests that only low level of resistance gain can be achieved with agents that evade PGP. To confirm that MDR1 expression is not driving resistance, we will assess the expression of this gene in parental cell lines and the resistant subclones, expecting to see no correlation between expression and resistance.

Another mechanism of resistance is by increased expression of βIII tubulin. This mechanism confers resistance to most microtubule targeting agents. However, compounds that disrupt microtubule polymerization by interacting with the colchicine binding site, such as FROST450, are typically not affected by βIII expression levels. We therefore expect that we will not see increased expression of βIII in cells with acquired resistance to FROST450. We also expect no βIII connection to FROST 450 resistance since FROST450 is not cross-resistant with taxanes, and taxane resistance can and does occur via βIII expression. As for MDR1, we will assess βIII tubulin expression in parental and resistant cell lines to confirm this expectation.

The most specific mutations for evading microtubule targeting agents are through point mutations in tubulin. Generally, those that arise to evade microtubule stabilizing agents, such as ixabepilone or taxanes, make microtubules more fragile and difficult to stabilize. Those that arise to confer resistance to microtubule polymerization inhibitors stabilize microtubules and make them more resistant to depolymerization. We expect mutations in tubulin genes that confer resistance to FROST450 to increase microtubule stability. Despite ixabepilone being a microtubule stabilizing agent that has the opposite effect of FROST450 on microtubules, mechanisms of resistance to ixabepilone provide an interesting template for understanding

mechanisms of FROST450 resistance. Both evade PGP and show limited cross resistance to taxanes. As with ixabepilone, high levels of FROST450 resistance in A2780 cells cannot be achieved; only low levels of resistance are reached, and even that resistance takes a significant time to emerge. Ixabepilone resistance in these cells first emerges as a result of point mutations in the class I β tubulin gene (TUBB1). Subsequent resistance levels are achieved as genes with the normal, non-mutated sequence are silenced and an increasing proportion of the protein product is expressed from the mutated gene. Our hypothesis is that resistance to FROST450 emerges from mutations in the class I β tubulin genes. We expect to find changes in the class I β tubulin open reading frame in cells with the lowest level of FROST450 resistance and to see that production of the normal, non-mutated version of this gene decreases in cell lines with higher and higher levels of resistance to FROST450. Since FROST450 and ixabepilone have different effects on microtubule polymerization and different binding sites in tubulin, we do not expect point mutations that confer resistance to ixabepilone to confer resistance to FROST450. Thus, we expect to find novel point mutations in the class I β tubulin gene in cells with acquired resistance to FROST450. Alternately, we may find point mutations that have been previously reported to confer resistance to other microtubule polymerization inhibitors, such as vinca alkaloids or colchicine.

SPECIFIC AIM 2: Identify cellular changes associated with acquired resistance to FROST450.

We expect that resistance mechanisms to FROST450 may result from cellular changes that operate to reduce or counteract the effect of the agent. These cellular changes may not be easily detectable by genetic sequencing. This aim will address these possible changes.

One hypothesis is that cells will counteract microtubule targeting agents by altering the stability of the microtubule cytoskeleton, allowing them to grow normally in the presence of a stabilizing or disrupting agent. As FROST450 inhibits tubulin polymerization in an act of destabilization, we expect that class I β tubulin mutations associated with FROST450 resistance could increase the stability of tubulin polymers. This is founded on the paradigm that microtubules have an optimal level of stability, where there exists an optimal balance between tubulin polymerization and depolymerization. Increasing the inherent stability of microtubules would allow them to achieve this optimal balance in the presence of a destabilizing agent like FROST450. We would then expect that FROST450-resistant subclones will require the presence of FROST450 to maintain optimal tubulin stability; they may grow more poorly in the absence of drug. Experimentally, we expect that these cells, with FROST450 dosing, will have a normal microtubule cytoskeletal morphology. However, when not in the presence of FROST450, we would expect the tubulin to be out of its optimal balance and in a more stable state, which would change the morphology of the microtubule cytoskeleton. As A2780 ovarian cancer cell subclones acquire resistance to higher and higher doses of FROST450, we expect any corresponding morphological changes when not in the presence of FROST450 to be most pronounced and persistent in the subclonal line resistant to the highest concentration of FROST450. We can test this by examining microtubule structure in FROST450-resistant cells grown in the presence or absence of drug by staining these cells with tubulin antibodies and visualizing the cytoskeleton by fluorescence microscopy.

Another hypothesis is that altering the balance of polymerized and unpolymerized tubulin induces cellular stress, and cells compensate for this by altering cellular activities associated with this stress. We have found that the presence of FROST450 potentially alters ATP production in cells. Further, it has been shown that increased levels of unpolymerized tubulin interferes with mitochondrial function, which would reduce metabolic efficiency of the cells and slow ATP production. We believe that cells could compensate for this by making alterations to cellular metabolism in mitochondria, either producing more mitochondria or altering the activity of existing mitochondria. Thus, we would expect there to be increasing numbers of mitochondria as resistance to FROST450 appears and increases. This can be tested by staining parental and resistant cell lines with MitoTracker and counting the number of mitochondria per cell for each line as visualized by fluorescence microscopy. We can also measure mitochondrial activity of each cell line as we lower, maintain, or raise the drug concentration. We would expect to see mitochondrial activity inversely correlate with the drug concentration for each cell line.

SPECIFIC AIM 3: Determine the resistance of FROST450-resistant ovarian cancer cells to other microtubule targeting agents.

As ovarian cancer cells acquire and increase in resistance to FROST450, it is possible that the molecular basis of resistance also confers resistance to other agents. Least likely is broad spectrum resistance including agents that are not microtubule targeting drugs. Given the lack of taxane cross-resistance we have previously documented, we also do not expect general cross-resistance with all or even most microtubule targeting agents. There is a likelihood of cross-resistance with a subgroup or specific instances of microtubule targeting agents, such as other polymerization inhibitors or other colchicine binding site compounds. To document the extent of any cross resistance, we will assess the potency of other microtubule targeting agents in parental A2780 cells and the isogenic FROST450-resistant subclones.

We will test agents that have the opposite effect on microtubule organization by testing the stabilizing agents paclitaxel (a taxane) and the ixabepilone analog epothilone B. We expect no cross-resistance to paclitaxel, given the previously discovered lack of resistance to FROST450 in taxane-resistant cells. This likely arises as the predicted tubulin mutations associated with acquired FROST450 resistance will alter the colchicine binding site, not the taxane binding site on the β aspect of the αβ tubulin dimer. Ixabepilone, albeit similar to FROST450 in that it also evades PGP, is a microtubule-stabilizer that also binds to the taxane binding site. We therefore expect similar results to that of paclitaxel, in that there is no demonstrably significant cross-resistance to ixabepilone.

We will also test agents that disrupt microtubules, including vincristine and eribulin. Vincristine and eribulin bind tubulin at distinct sites on the β tubulin monomer, both from each other and from FROST450. Both also have vastly different resistance profiles (they are susceptible to resistance from βIII tubulin expression, for example, and thus have cross-resistance with taxanes). We expect FROST450-resistant cells will demonstrate no cross-resistance to vincristine nor eribulin.

Finally, we will test whether cross resistance emerges with agents that bind tubulin in the same location as FROST450. Since FROST450 binds in the colchicine binding site, colchicine is a clear choice. Indibulin binds tubulin in the colchicine binding site in a manner that overlaps significantly with FROST450 and will also be tested. Interestingly, it also evades mechanisms of taxane resistance, like FROST450. Tubulin mutations acquired from FROST450 resistance are likely to affect the activity of other compounds if they bind in the same location, so we expect significant cross-resistance with both colchicine and indibulin.

If there is a lack of cross resistance between FROST450 and other microtubule targeting agents tested, we would expect parental and FROST450-resistance subclones of A2780 cells to exhibit the same concentration-dependent response (EC50) to the test agent. If there is cross resistance of FROST450 with the other agent tested, we would expect FROST450-resistant subclones of A2780 cells to have a higher EC50 for the test agent than the parental cell line. In short, we expect the FROST450-resistant cells to survive higher doses of the other agent if there is cross-resistance. It is possible that FROST450 resistance increases the susceptibility of cells to other agents, particularly microtubule-stabilizers that have the opposite effect on microtubule dynamics (taxanes and ixabepilone). Here we would expect FROST450-resistant cell lines to respond to lower doses of these agents than the parental cell line; the EC50 of the FROST450-resistant subclones will be lower than that measured for parental cells.

The purpose of this specific aim is to determine the cross-resistance of FROST450-resistant subclones against other antimitotic agents. This will reveal how to position FROST450 in the clinic in relation to approved microtubule targeting agents.

Project Timeline

Task	Projected Date Completed
ATPlite cross-resistance assays and data processing	March 21st
Morphological staining and analysis	March 30th
cDNA sequencing and analysis	April 20th
Honors thesis written	May 31st
Submitted for publication in research journal	August 1st

Faculty Qualifications

Dr. Marc Hansen:

He is the faculty advisor for the project. He graduated from BYU with a BS in Molecular Biology, after which he earned a PhD in Cancer Research from Stanford. His research experience has involved studying mechanisms of cancer metastasis and epithelial-mesenchymal transitions, as well as drug discovery and development with various biomedical startups with which he has been involved. The compound of interest in this thesis research, FROST450, is one

discovered and modified by Dr. Hansen's Frost Biologic company. He is well-versed in cancer mechanisms, FROST450 function and use, and the various assays being used to characterize resistance to the antimitotic compound, making him qualified to advise the project. I work in his lab and with other research assistants in his lab to run and complete assays for the project.

Dr. Stephen Piccolo:

He is faculty reader for the project. He graduated from BYU with a BS in Management Information Systems, followed by a PhD in Biomedical Informatics from the University of Utah. He has expertise in analyzing biomedical information and data systems, which makes him qualified to review and aid in the DNA sequence and mutation analysis for the project. I have not personally been in his class or worked in his lab. However, he knows and has worked with Dr. Marc Hansen and I have had meetings with him regarding my thesis research, and he is willing to be involved as faculty reader.

Culminating Experience

My goal for this thesis research, which is building upon and is a culmination of previous research we have performed in Dr. Marc Hansen's lab, is publication in a cancer research journal, such as Cancer Cell.