The Vault Project:
Down-regulation of vault RNA expression via iRNA technology leads to increased drug sensitivity in small cell lung cancer.

by
Katina N. Moore

Dr. Carolyn N. Vann
Honors Thesis Advisor
Department of Biology

Ball State University
Muncie, IN 47306

Submission Date: May 8, 2004
Graduation Date: May 8, 2004
Abstract:

Small-cell lung cancer cells, GLC4, that confer a multi-drug resistance (MDR) phenotype are resistant to the chemotherapeutic drug doxorubicin. Concurrent with MDR is an upregulation of cytoplasmic vaults, ribonucleoprotein particles that have a proposed function in nuclear-cytoplasmic transport. GLC4 cells were subjected to interfering RNA (iRNA) treatment that down-regulated expression of one of the RNA species (vRNA1) associated with the vault complex. These cells, termed psi-VRNA1, along with appropriate control cells were treated with low (345 nM) and high (1035 nM) concentrations of doxorubicin for 6 hrs, 12 hrs, and 24 hrs and assessed for apoptotic activity.

Preliminary data suggest that destabilization of the vault structure reestablishes GLC4 sensitivity to doxorubicin. A caspase-8 colorimetric assay (Sigma) was utilized to gauge apoptotic activity. At the 6-hour time point a slight increase in apoptosis was observed in the psi-VRNA1 cells, which no longer expressed functional vaults, when compared to GLC4 normal controls. Most interestingly, at 12-hours a maximal apoptotic response was seen at the lower concentration of doxorubicin. After 24-hours of treatment with doxorubicin the same response is not observed. In this preliminary investigation, the data suggests that a 12-hour, 345 nM exposure of doxorubicin provides the optimal treatment regime to induce maximal levels of apoptosis.

Acknowledgements:

It is most fitting to first extend sincere appreciation and gratitude to Dr. Carolyn Vann. After one class with her, she was able to match me with an exciting project that
was the “perfect fit”. In the Vann lab, I was provided a unique experience that allowed me to be an independent thinker while maintaining the student-mentor relationship. In addition, I had ample access to opportunities to which I would have never been exposed. Principally, I was expected to present and defend my work to the scientific community; a feat I would have never pursued on my own. As a result, I can more confidently converse about scientific methodology. I most appreciative to Dr. Vann, not for the scientific protocols she introduced me to in the lab, but for the guidance I received that can be applied to any aspect of my career. This experience has given me a great foundation for long-term success.

In addition, it is appropriate to acknowledge Dr. James B. Olesen who very graciously served as scientific support system throughout this entire project. He was very willing to dedicate and share lab protocols, supplies, and time to our lab efforts. Mike Adam, a graduate student, played very integral role in the project as well. He was a wonderful resource in the lab for troubleshooting and moral support.

Behfar Ardehali, a previous Ball State graduate student, must also receive acknowledgement because he initially pursued the vault project. He made my undertaking of a graduate project as an undergraduate feasible. Even though he is pursuing his doctorate at another university, he did not hesitate to answer in questions I asked of him concerning his work at Ball State.

The Biology Department as a whole was very receptive and supportive to both our successes and failures in this project. The collaborative effort displayed here is one that I hope to mimic in my future career.
Introduction:

As cancerous malignancies provide a great challenge to the human life expectancy, resistance of these cancers to chemotherapeutic treatment has become a growing dilemma. Small cell lung cancers that display this phenomenon called multi-drug resistance (MDR) have been shown to actively pump doxorubicin (a commonly used chemotherapy drug) out of the nucleus. In this event, the drug, which has nuclear targets, is rendered ineffective because it is prevented from remaining in the nucleus where it is active. Consequently, abnormally high levels of vault structures have been shown to be produced in such cells that exhibit an increased drug resistance. This observation serves as a strong suggestion the vault particle functions in conferring a multi-drug resistant phenotype to cancer cells.

Previously, human small-cell lung cancer cells from the cell line GLC4, which confer a multi-drug resistance (MDR) phenotype when exposed to doxorubicin, were subjected to interfering RNA (iRNA) technology. This technology produced a treatment effect that down-regulated expression of one of the RNA species (vRNA1) associated with the vault complex in human cells. These cells having destabilized vault RNA are termed psi-VRNA1. They along with appropriate control cells were treated with low (345 nM) and high (1035 nM) concentrations of doxorubicin for 6 hrs, 12 hrs, and 24 hrs and assessed for apoptotic activity.

Review of Literature:

Vaults have been well characterized in the two decades since their discovery (1). At basal levels vaults are abundant, with 10,000 vaults reported to populate one differentiated cell (2). Kickhoefer et. al have made great strides in answering the
questions surrounding vault function. They have not only demonstrated that vaults are abnormally abundant in cells possessing multi-drug resistance (MDR) (3), but have also characterized the protein structural components of the vault ribonucleoprotein. These proteins include the major vault protein (MVP); TEP1, a telomerase protein subunit; and vault poly(ADP-ribose) polymerase (VPARP). In addition, an interaction of the 50-kDa La-binding protein with the vault complex has been newly identified (4).

Although its function has not been specifically defined, the structural complexity of the vault particle is consistently being supported. Its composition of both protein and RNA proves to be one of its most distinguishing characteristics. While the protein components seem to provide structural stability to the complex it is thought that the vault RNA plays the role as the functional unit of the vault structure (5). In human cells, vaults are composed of three separate RNA species: vRNA1, vRNA2, and vRNA3.

Octagonal in nature, vaults have an open or flowering conformation that is large enough to accommodate the large size of an intact ribosome (6). Interestingly this octagonal shape of the vault is analogous to the pores within the nuclear membrane. This observed structural similarity between the vault complex and the nucleus provides evidence that strongly suggests an involvement of vaults in some form of nuclear transport.
**Materials and Methods:**

**Cell Culture**

The small cell lung cancer cell line, GLC4/REV, previously utilized by Behfar Ardehali was used (7). The cells of this GLC4 population display the MDR phenotype and also abundantly express vault particles upon exposure to chemotherapeutics. The GLC4/REV cells are a revertant population stemming from a GLC4/ADR population that was donated by Dr. Valerie Kickhoefer from the Biological Chemistry Department at UCLA. GLC4/ADR cells depend on consistent drug supplementation for growth in vitro. The non-dependent GLC4/REV cells are grown in culture using drug-free RPMI 1640 media that contains 10% Fetal Bovine Serum (FBS) and supplements of L-glutamine and antibiotic/antimycotic.

**Freezing Cells**

Cells were preserved and frozen in a freezing media that contained 7 parts of RPMI media (supplemented with L-glutamine); 2 parts FBS; and 1 part DMSO (dimethylsulfoxide). In a final step the freezing media was filter sterilized using a 0.2 µm filter-tip syringe. To obtain a final concentration of $1 \times 10^6$ cells per ml of freezing media, the cell-media mixture was centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in sterile freezing media. In freezing vials 1 ml aliquots of cells were distributed and the vials were placed in ‘Mr. Frosty’ containers overnight at −80°C. The following day the frozen vials were placed in the liquid nitrogen tank for long-term storage.
**Thawing Cells**

The freezing vials were promptly placed in a 37°C water bath and thawed when cells were needed for culture. As a washing step, warm RPMI media (5 ml) was added to the thawed cells in a dropwise fashion. Centrifugation under the aforementioned settings was completed to pellet the cells and the supernatant was removed. Another 10 ml of warmed medium was used to resuspend the pellet and the whole mixture was transferred to a 25 ml cell culture flask. The cells were grown in an regulated incubator set to a 37°C temperature and a 5% CO₂ level.

**Cell Populations**

Four populations of cells were maintained. Three groups of GLC4 cells were used (psi-vRNA1; psi-Neg, and GLC4/REV) and one group of AKR-DP-603 cells was used to complete this investigation. The GLC4/REV cells represented the normal, cancer cell population that displays MDR and an upregulation of vault particles upon exposure to doxorubicin. The psi-vRNA1 cells were subjected to iRNA technology, which served to destabilize the vRNA1 component of the vault structures in these cells. These cells that represent the experimental cell population were transfected with a plasmid vector containing the interfering RNA sequence specific to the vRNA1 gene sequence. The psi-NEG serve as an iRNA control needed to highlight the non-specific activity of the RNA interference. These cells were transfected with a plasmid vector that contained an interference sequence with no known specificity to any human gene sequence (provided in the kit).

As cells that in vitro are non-adherent and grow in suspension like the GLC4 cells, AKR cells served as an overall experimental control. These cells, stemming from a
mouse T-cell, were maintained using a different medium termed DP medium. All of the described protocols for freezing and thawing cells were followed, but DP medium was used instead of RPMI.

As aforementioned, the GLC4 cells do not adhere to the surface of the culture flask as they grow. They typically form grape like clumps as they replicate in the culture media. The GLC4 cells rapidly consumed the available nutrients present in the media, but 70-80% confluency was achieved following about 36 h of growth. The AKR cells grew more slowly, and formed larger, more densely populated islands within the culture media.

**Treating Cells**

Cell treatments were performed using the chemotherapeutic agent doxorubicin. Treatments were conducted at two concentration levels: a low (345 nM) concentration and a high (1035 nM) concentration. The original sample of doxorubicin (Sigma, St. Louis, MO) was completely dissolved (1mg/ml) and then diluted to a 400 μg/ml stock concentration. Of this stock concentration, 17.1 μl were used to treat at a 345 nM concentration and 51.3 μl were used to treat at a 1035 nM concentration. Doxorubicin treatments were pipetted directly into the culture flask containing 7.5 x 10⁵ cells and the appropriate cell culture media.

**Protein Extraction and Determination**

After the four cell populations were treated with doxorubicin, whole cell lysates were collected from each treatment group. Cold temperatures play an important role in inhibiting proteases (protein degraders), so the protocols involving protein lysates were all conducted on ice or at 4°C. At the conclusion of the drug treatment the cell medium
was immediately transferred into a 15 ml conical tube and centrifuged at 1,500 rpm for 5 min to pellet the cells. Following aspiration of the supernatant, the cells are resuspended in 1 ml of RIPA buffer and allowed to incubate on ice for 20-30 minutes.

To make a 50 ml portion of RIPA buffer the following components were added: 1.5 ml of 5M NaCl; 0.5 ml of 1M Tris (pH 8.0); 0.5 ml of 10% SDS; 0.5 ml IGEPAL CA-630; 0.25 g of NA-deoxycholate; and, distilled water (or PBS) to a volume of 50 ml. All of the components were blended and dissolved with stirring and the final solution was stored at 4°C.

Following the incubation period on ice, the cell suspension was transferred to a pre-chilled homogenizer where at least 60 strokes were applied to ensure that the cells were ruptured and proteins released. From the homogenizer, the homogenate was transferred to a pre-chilled microcentrifuge tube and then centrifuged once more for 10 min (~14,000 x g) to pellet the non-protein cellular components. Following this step the supernatant was retained, removed from the pellet, and stored at −80°C. (It might be beneficial to divide the supernatant into several aliquots so as to avoid protein degradation from repeated freeze-thawing.)

Once the protein had been extracted the amount of protein available in the sample was quantified. This protein determination was conducted using the microplate protocol supplied by BioRad, Hercules, CA. First, a BioRad protein standard (~1.47 μg/μl) was used to create a standard curve correlating spectrophotometric absorbance with a known concentration of protein. The standard curve was completed using 5, 10, 20, 30, and 40 μl of the known standard in a total of 100 μl. Subsequently 10 μl of each dilution was pipetted into individual wells in a 96-well round bottom plate. For each of the unknown
samples, 3 μl of protein lysate was diluted with 7 μl of deionized water to attain a total volume of 10 μl. After 10 μl volumes of protein were pipetted into the appropriate standard or unknown wells, 200 μl of the BioRad protein reagent was added to each. The protein reagent was prepared by diluting the concentrated dye reagent 1:5 in deionized water.

To determine how much of the protein lysate to use in this determination, a standard series with the lowest and highest concentration of known protein was prepared in microfuge tubes and the dye reagent was added to produce the characteristic blue color that is vivid when protein is present in the sample. Several lysate samples were chosen and arbitrary aliquots were taken and diluted to a total volume of 10 μl. Dye reagent was added to each and the resulting blue color was compared to that of the standards. The microliter amount of sample that gave a blue color less intense than that of the highest standard was used. For this particular determination, 3 μl was optimal to provide a blue color within the range of the standard curve.

Once the dye reagent was added to each sample, it was allowed to incubate 5-10 minutes and was then read on the Stat Fax-2100 automatic microplate reader. A standard curve correlating the absorbance values at 495 nm with the amount of protein was drawn using the Microsoft Excel program and used to determine the concentration of the unknown samples. From this determination the total amount of protein within each whole cell lysate was determined.

**Caspase-8 Colorimetric Assay**

To analyze how much of the apoptotic protein, caspase-8, was present in each sample, a caspase-8 colorimetric assay (Sigma) was used. Based on a microplate
technique, this kit spectrophotometrically measured the how much of the caspase-8 chromogenic substrate (Acetyl-Ile-Glu-Thr-Asp-p-Nitroaniline) was cleaved in each unknown sample. To conduct this assay, 200 µg of protein from each lysate was combined with 1X assay buffer (provided by the kit) to a final volume of 90 µl. An additional 10 µl of chromogenic substrate was added to each sample to raise the entire reaction volume to 100 µl. Each unknown sample was assayed in triplicate. To reduce pipetting errors and variability a master mix was prepared for each sample that provided reaction sample enough for three reaction wells. The 600 µg of protein and 1X assay buffer appropriate for each sample was added together in a pre-chilled eppendorf tube and allowed to sit on ice. When all of the sample were prepared, 30 µl of caspase-8 substrate was added to each tube and gently vortexed. Following a quick spin, 100 µl of the reaction mixture was aliquoted into each well.

A positive control for caspase-8 activity was prepared by diluting with 10 µl of a pure caspase in 80 µl of 1X assay buffer. To this another 10 µl of chromogenic substrate was added and allowed to react. In addition, a positive control with an added inhibitor was included in the assay to indicate the capacity of substrate for non-specific hydrolysis. For this reaction, 2 µl of a 25 µM caspase-8 inhibitor was added to the same amount of positive control mentioned above and allowed to incubate for 10 minutes on ice (78 µl of assay buffer also included). Following this incubation, caspase-8 substrate was added. A well containing the reagent blank (90 µl 1X Buffer and 10 µl substrate) was included (well A1).

When all of the reaction wells were prepared in the 96-well plate, it was covered with parafilm and incubated at 37°C for 2 hours. Following the incubation, the
absorbance was read at a wavelength of 405 nm and the amount of substrate hydrolyzed in each sample was determined.

To correlate the absorbance at 405 nm with the amount of p-Nitroaniline (p-NA) released by the substrate within each sample, a calibration curve was generated using standard p-NA. Amounts of standard p-NA ranging from 0-20 nmol were diluted with 1X buffer to the reaction volume. In a similar manner executed during the generation of the protein standard curve, a p-NA calibration curve was produced and utilized to determine the concentration of p-NA produced in each unknown sample. All calculations and graphing for the caspase-8 assay were conducted using the Microsoft Excel program.

ANOVA statistical analysis was completed using MINITAB 14 software. The triplicate data values were transferred into the program from Excel. From the STAT menu the one-way ANOVA was chosen. Samples for which comparative analysis was required were identified and a Tukey’s pairwise comparison was selected simultaneously (family error rate of 5). From the output data, the p-value was observed.

Results:

This investigation, operating at exponential proportions, included two levels of treatment (345 nM and 1035 nM); three time points (6, 12, 24 hours); and four cell populations. For each cell population, in vitro treatments with doxorubicin were completed at each concentration for each time designation described.
**Hygromycin Treatment**

Both the psi-vRNA1 cells and the psi-NEG cells were created through the use of iRNA technology and, thereby, subjected to plasmid transfections. To verify that both these GLC4 cell line variants were still undergoing the cell cycle as stable transfects, each culture was subjected to a hygromycin treatment. The plasmid vector used to in the transfection of these cells contained resistance genes to the drug hygromycin. If the cells being cultured had, indeed, remained stably transfected, then the cells would continue growth under selective treatment with hygromycin. The psi-vRNA1 and the psi-NEG cells were treated with a 0.05 mg/ml concentration of hygromycin for 96 hours.

After two-days of growth in the presence of hygromycin, neither cell population displayed the characteristic confluency, but the remaining cells appeared very healthy and unaffected by drug treatment. The cells were pelleted (1000 rpm, 5 minutes) to remove any dead cells and old media, and the pellet was resuspended in fresh media containing 10% FBS.

**Protein Determination**

Because of the very large sample volume, it was most efficacious to use the microplate technique described. Because a strong blue color resulted when only microliter amounts of lysate were used, it was evident early in the determination process that the whole cell lysates were very concentrated with protein. The standard curve produced (Figure 1), as expected, was linear plot with a 0.98 $R^2$ value.

**Caspase-8 Colorimetric Assay**

Preliminary data suggest that destabilization of the vault structure reestablishes GLC4 sensitivity to doxorubicin. Reported as fold increase of p-NA, the data resulting
from the caspase-8 assay indirectly indicates how much apoptosis occurs within each treatment group. At the 6-hour time point both treatment levels seem to display a slight increase in apoptosis was observed in the psi-VRNA1 cells (no longer expressing functional vaults) when compared to GLC4 normal controls (Figures 3). After 24-hours of treatment with the lower concentration of doxorubicin (Figure 5) the slight response observed at 6-hours is mimicked. At the higher doxorubicin concentration (Figure 5) the psi-vRNA cells express an unexplained dip in fold increase, which may be indicative of harsh treatment conditions characterized by the longest exposure at the highest treatment level.

However, most interestingly, at 12-hours a maximal apoptotic response was seen at the lower concentration of doxorubicin (Figure 4). In this preliminary investigation, the data suggests that a 12-hour, 345 nM exposure of doxorubicin provides the optimal treatment regime to induce maximal levels of apoptosis.
**Fig. 1**- Protein Standard Curve generated using Biorad Standard Protein (~1.47 μg/μl). The equation of this linear plot was used to precisely determine the concentration of protein in each unknown sample. Absorbance was measured at 595 nm.

**Fig. 2**- As with the Protein Standard Curve above, this pNA Calibration Curve depicts a linear plot that correlates the concentrations of the p-Nitroaniline standards with the absorbance values obtained at 405 nm.
**Fig. 3** - GLC4 cells treated with low (A) and high (B) concentrations of doxorubicin for an exposure time of 6-hours. The fold increase of the cell populations that underwent siRNA technology is shown in comparison with the normal lung cancer and the caspase-8 positive control. A slight increase of p-NA cleavage is visualized for both treatment levels.
Fig. 4- GLC4 cells treated with low (A) and high (B) concentrations of doxorubicin for an exposure time of 12-hours. At 12-hours the maximal p-NA cleavage is visualized at a lower concentration of doxorubicin for the psi-vRNA1 cells. At the higher concentration of doxorubicin, this same response is not seen. Highly evident is the large non-specific response seen in the psi-Neg population that was subjected to iRNA technology targeting a gene with no known specificity.
Fig. 5- GLC4 cells treated with low (A) and high (B) concentrations of doxorubicin for an exposure time of 24-hours. A markedly lower fold increase was displayed at 24-hours, especially at the higher concentration of doxorubicin. The psi-vRNA1 population experiences a dip in p-NA release when exposed to our experimental extremes (both the higher concentration of doxorubicin and the longest treatment exposure time). Very interestingly, in a previous caspase-8 assay a similar dip was seen observed in the psi-vRNA1 cells (data not shown).
Statistical Analysis

The ANOVA statistical analysis that was completed gave output data that indicated that our results were not statistically different. This was determined upon investigation of the P-values. To establish a statistical difference the p-value must be below a factor of 0.05. The P-values for each of the experiments ranged from 0.098 to 0.837 (Table 1).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>P-value (∼&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr, 345 nM</td>
<td>0.337</td>
</tr>
<tr>
<td>6 hr, 1035 nM</td>
<td>0.837</td>
</tr>
<tr>
<td>12 hr, 345 nM</td>
<td>0.098</td>
</tr>
<tr>
<td>12 hr, 1035 nM</td>
<td>0.469</td>
</tr>
<tr>
<td>24 hr, 345 nM</td>
<td>0.179</td>
</tr>
<tr>
<td>24 hr, 1035 nM</td>
<td>0.369</td>
</tr>
</tbody>
</table>

Table 1: The ANOVA statistical analysis determined that there was no significance among the treatment groups. P-values must be below 0.05 to be considered significant. It is interesting to note that the lowest p-value obtained was in the 12 hr-345 nM treatment group in which the psi-vRNA1 population seemed to display a maximal apoptotic response.

Discussion:

Upon evaluation of the caspase-8 assay data, it seemed as if an affirmative link between vaults and the MDR arena was uncovered. Although some convincing trends seemed evident, the statistical analysis suggests that none of the effects observed are of statistical validity. Despite efforts to minimize pipetting errors and to optimize the plate reader, a noticeable amount of variability was still recorded among the triplicate samples. This variability the most likely reason the data was not statistically valid.
When this data is compared to that previously done by the former graduate student, Behfar Ardehali, a great variability among the replicates is seen in both assays. The only distinction observed between the two assays is that differences in fold increase were greater among treatment groups achieved previously by Mr. Ardehali. With his data, not all groups were proven to be statistically different, but this was more than seen with the secondary assay. Some change in expression is still suggested by the data, but without strong statistics it cannot be unequivocally argued that vaults are a major player in MDR. It is possible that while they do play a role in MDR, it is not a primary role.

It is also possible that our experimental protocol needs to be revamped. The use of another caspase assay or a completely different apoptotic assay may be necessary. The caspase-8 protein occurs very early in its apoptotic cascade and it is a possibility that it does not serve as the best indicator of programmed cell death. Further experiments are underway that will once and for all elucidate whether or not vaults are at all critical in MDR. A similar experimental protocol is being pursued that will investigate the silencing of the vRNA3 construct in this human lung cancer. Data will indicate the relative importance of both vRNA3 and vRNA1 as it relates to the function of the vault.
References:


