

THE EFFECTS OF VERTEPORFIN
ON NON-SMALL CELL
LUNG CANCER

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DEDICATION

I would like to dedicate this to my parents. Without their love and support, I would never have been able to generate this work.

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Without the help of my mentor, Dr. Lawrence Quilliam, this project would have never come to fruition. His dedication and endless help have helped me develop to better analyze and go about answering biochemical questions. I would also like to mention the other members of my committee, Dr. Mark Goebel and Dr. Clark Wells. Their advice and support helped me to write this thesis and better articulate my thinking on my experiments. Members of other labs that I would like to mention are Brandon Lane and Lauren Bringman, They helped me troubleshoot and think of other methods to go about answering my questions. Lastly, I would like to mention my co-workers and scientists that I work with at Covance. Their input and opinions helped me to think critically about my experiments and extract the most amount of information from my data.

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Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancers and is the leading cause of cancer death in the United States. Better treatments must be devised in order to improve the prognosis of this disease. Verteporfin, an FDA approved drug, has recently been reported to downregulate a potential core pathway of NSCLC, the Hippo pathway. The pathway consists of a kinase cascade to control the transcriptional coactivators YAP and TAZ. When these transcriptional coactivators lack phosphorylation of key residues, they are able to translocate into the nucleus and bind to the TEAD member of transcription factors. This augments transcription for genes responsible for proliferation, survival, and stem maintenance. In this study, we report that verteporfin limits proliferation and survival of NSCLC and may potentially be a viable treatment option. Inhibition of cell survival dose-dependently correlated with inhibition of YAP-TEAD transcription target CTGF. We also report the covalent homo-oligomerization of p62, a prominent protein involved with autophagy, with the introduction of verteporfin into NSCLC cells.

Lawrence A. Quilliam, Ph.D., Chair

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ABBREVIATIONS

ALDH	Aldehyde Dehydrogenase
CTGF	Connective Tissue Growth Factor
dPBS	Dolbesco's Phosphate Buffered Solution
MTT	3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	Non- Small Cell Lung Cancer
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TAZ	Transcriptional Co-activator with PDZ binding motif
YAP	Yes-associated Protein

INTRODUCTION

Non-small cell lung cancer (NSCLC), accounts for 85-90% of lung cancers. According to the American Cancer Society “Lung cancer accounts for about 27% of all cancer deaths and is by far the leading cause of cancer death among both men and women. Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined.”(1) Finding a new way to treat this cancer and improve prognosis is of great need. A signaling pathway that may prove effective in targeted drug therapy for NSCLC is the Hippo pathway. The Hippo pathway consist of a series of kinases that regulate the transcriptional co-activators Yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1/transcriptional coactivator with PDZ binding motif (TAZ). These transcriptional co-activators can go on to activate members of the TEAD transcription factor family, and potentially other transcription factors, to control proliferation, survival, and stem cell maintenance (2-4). Targeting this pathway may prove to be very promising in the treatment of lung cancer.

Visudyne (Verteporfin), an FDA approved drug for the treatment of macular degeneration, has been shown to have a negative effect on TEAD downstream transcription. The current understanding of how verteporfin accomplishes this is through preventing the YAP-TEAD complex from forming [5]. Hence, treatment with verteporfin could effectively treat cancers with aberrant Hippo activity. NSCLC cell growth has been shown to be inhibited by overexpressing MST1, preventing YAP from acting as a transcriptional coactivator (5). From this it can be hypothesized that verteporfin would be effective in the treatment of lung cancer.

The Hippo Pathway

The Hippo pathway is a fairly recently identified pathway, with the orthologs being first discovered in 2002 in *Drosophila melanogaster*. The pathway was found to be highly conserved in mammals and to be a major controller of organ size, which was unprecedented in other pathways (4). The elicited response to mutation in any one of the upstream kinases in the Hippo pathway generates an overgrowth of the targeted organ. These mutations diminish the kinases function to phosphorylate, ultimately inactivating the Hippo pathway. This inactivated form allow for increased proliferation and cell growth akin to that of a tumor (2).

The mammalian Hippo pathway begins with the protein complex of MST1, MST2, and SAV1. When the pathway is active, the phosphorylated MST1/2 complex phosphorylates the complex of large tumor suppressors 1 and 2 (LATS1/2) and MOB1. This in turn activates the LATS1/2 kinases to phosphorylate YAP/TAZ. This event keeps YAP and TAZ sequestered in the cytosol, preventing them from acting upon transcription factors in the nucleus. Depending on the phosphorylation site on YAP and TAZ, it will either be retained in the cytoplasm or marked for degradation. There are five known sites for LATS1/2 to phosphorylate YAP, 4 of these being conserved in TAZ. A phosphorylation event of YAP on serine 127 creates a 14-3-3 binding site, which will keep it retained in the cytoplasm. Subsequent Phosphorylation of serine 381 triggers ubiquitination and proteasomal degradation of YAP (3,6). However, if the pathway is never initiated, YAP and TAZ will be in their active unphosphorylated form and be able to cross the nuclear membrane to act as transcriptional co-activators.

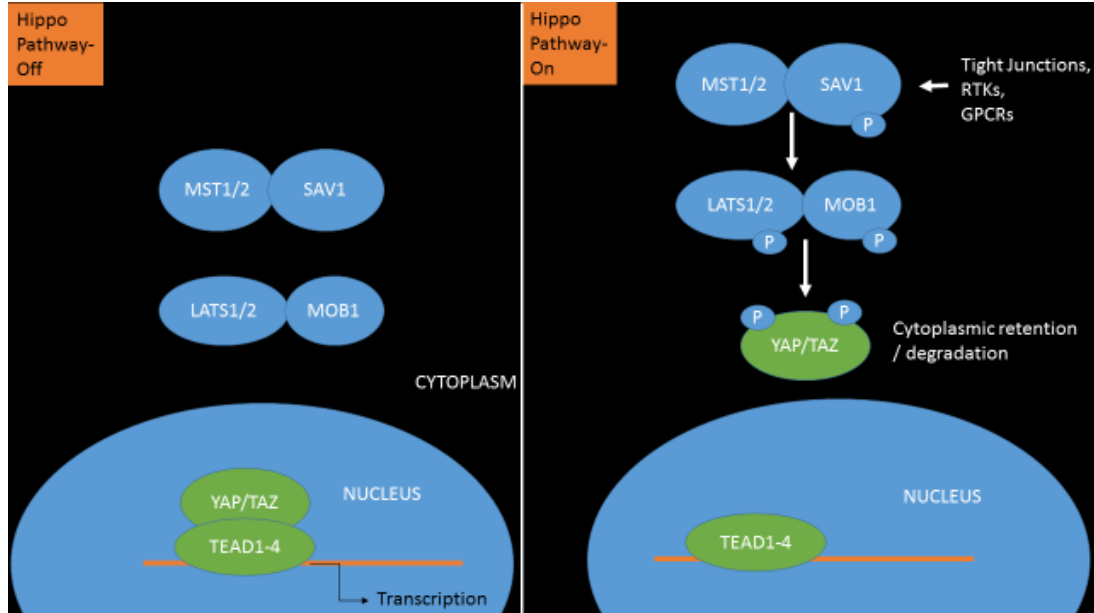


Figure 1. Depiction of the core cascade of the Hippo pathway in its active and inactive states. In active Hippo signaling, the phosphorylated MST1/2-SAV1 complex go on to phosphorylate LATS1/2, which in turn phosphorylate YAP/TAZ. This event keeps YAP from translocating into the nucleus and act as a transcriptional co-activator of TEAD1-4. In inactive Hippo signaling, the phosphorylation cascade never occurs, allowing YAP/TAZ to act on TEAD1-4 to modulate transcription.

Upstream signaling of the Hippo pathway is poorly understood. The core kinase cascade of the Hippo pathway is controlled by a multitude of factors that all go into sensing the physical state of the cell. A very prominent feature of the Hippo pathway is its interconnection with other cellular pathways (7). This interconnectedness allows the Hippo pathway to sense and respond to energy levels, cellular contact, growth factors, and polarity of the cell. In tumor conditions, these interconnections may funnel some of the classical phenotypes seen in tumors through the Hippo pathway; making it a great potential therapeutic target.

Verteporfin

Verteporfin, trademarked as Visudyne, is currently used in the photodynamic treatment of age related macular degeneration. It is composed of two regioisomers (same chemical formula, but a functional group changes position on the parent molecule), which are listed below. The drug is activated at a 690nm wavelength, at which point singlet oxygens are produced while in the presence of oxygen (8). After its FDA approval, it was also found to suppress the oncogenic activity of YAP without the need for light activation (9). To demonstrate whether verteporfin binds to YAP or TEAD, Liu-Chittenden et al mixed verteporfin with purified versions of the proteins. The author found that verteporfin coeluted with YAP, signifying verteporfins binding partner is in fact YAP.

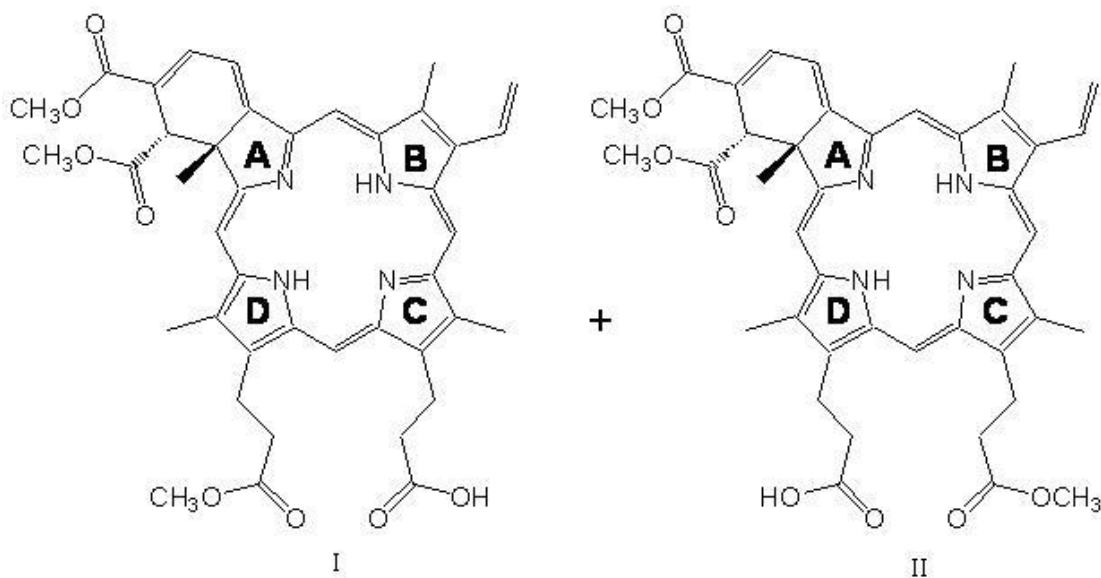


Figure 2. The two regioisomers of verteporfin.

Verteporfin is known to produce singlet oxygens with exposure to light, but these reactive species appear to be produced at modest levels even without light exposure (10,11). Singlet oxygen is a short-lived electrophilic molecule that reacts rapidly with unsaturated carbons, neutral nucleophiles and anions (12,13). Verteporfins basal production of singlet oxygens causes damage to proteins near the site of where it was formed. Consequently, some proteins may become non-functional or aggregate protein crosslinks may arise. This is problematic for potential therapeutic value, as proteotoxicity may be unavoidable. On the other hand, this could be a very useful secondary function of verteporfin. Some studies have reported normal cells are able to clear out the proteotoxic effects of verteporfin while tumor cells cannot (11,14). If this is the case, it adds more credence to verteporfin being an effective cancer treatment.

Another interesting phenomenon cells exhibit when exposed to verteporfin is the lack of autophagy (10). This phenotypic change can be attributed to the polymerization of p62 with itself. The protein p62, also known as sequestosome 1 (SQSTM1), is a multifunctional adaptor protein that recognizes and loads ubiquitinated cargo into an autophagosome (10,15). The singlet oxygens produced basally by verteporfin cause this multimerization of p62. Which is also a downward slope, as more p62 polymerize, less autophagic degradation occurs, allowing more damaged proteins to build up. This may also be a major proponent of cell death with verteporfin treatment.

METHODS

Cell Passaging

Stock plates of cell lines were passaged every 3 days for the H460 cells and 3 to 4 days for H23 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Medium was aspirated off the stock plates and replaced with 10 mLs of dPBS. dPBS was then aspirated and replaced by 1 mL of trypsin/EDTA solution, followed by being placed into a cell culture incubator for 5 minutes. Afterwards, 9 mLs of media was dispensed onto the cell suspension to inactivate the trypsin. The suspension was then agitated by gently pipetting up and down several times to ensure all cells are detached from the plate and each other. Cells were then quantitated by pipetting 10 uLs onto both sides of a hemocytometer and counting five squares on each side. Stock plates were set at 200,000 – 300,000 cells per 10 cm dish.

Clonogenic Assay

To assay for survival and proliferation, clonogenic assays were conducted. Cells were diluted to 167 cells per mL of media. Three mLs of the diluted cell suspension was dispensed onto a 60 mm plate for approximately 500 cells per plate. Plates were then incubated in a cell culture incubator for 24 hours. Media was aspirated and replaced with fresh media infused with verteporfin concentrations listed below. Cells were left to incubate for 72 hours. The media was then aspirated off and the dish was washed twice with 3 mLs of dPBS. Following aspiration of the dPBS, fresh media was placed into the dish and placed back into the cell culture incubator. At this point the cells were left to recover from the exposure to verteporfin. The recovery process takes 7-9 days. During this period, the media was changed out by day 4. Once the colonies on the control plate were visibly noticeable, the plates were processed for staining. Media was aspirated and plates were washed with 4 mLs of dPBS. After aspirating the wash, 3 mLs of 10% acetic acid / 10% methanol was pipetted onto the plate as a fixative. The plates were then incubated for 20 minutes followed by removing the fixative. Crystal violet was then used to stain the cells for visualization and potential colony counting. One mL of crystal violet was pipetted onto each plate and let sit for 20 minutes. The residual crystal violet is then aspirated off of the plates and washed under a low-pressure stream of water. The wash was performed 4-5 times or until all of the residual crystal violet is washed away. The stained colonies are then visualized and interpreted for changes in survival and proliferative capacity.

MTT Assay

Measurements of cell viability and proliferation were also acquired utilizing MTT assays. This assay assesses cells metabolic activity via oxidoreductase enzymes reducing the tetrazolium dye MTT to formazan. Cells were set on a 96 well plate at 2000 cells per well. The plates were incubated for 24 hours at 37° C. Media was then aspirated and replaced with media infused with drug concentration of choice, in triplicates. Plates were then incubated for ~72 hours in the cell culture incubator. 10 uLs of MTT Reagent was added to each well and incubated for ~4 hours. Media was aspirated and 100 microliters of alcohol/detergent reagent was added to each well to dissolve the precipitate. After 3 hours of incubation in the dark, the plate was then quantified on a spectrophotometer, recording at an absorbance of 570 nm.

Matrigel Assay

Matrigel assays were conducted to view cell morphology and phenotype changes with the addition of verteporfin in three-dimensional culture. Matrigel was thawed to 4°C, 200 uLs was pipetted onto a 40 mm dish and spread evenly across the center of the plate with a pipette tip. Plates were then incubated in a cell culture incubator for 20 minutes to allow the Matrigel to set. During this time cells were trypsinized and counted. 200,000 cells were dispensed on top of the Matrigel and let set for 24 hours. Media was aspirated and replaced with verteporfin infused media. At periodic intervals, microscopic images were collected of each set of plates at 4X or 10X magnification to discern any phenotypic changes with the cells.

ALDEFLUOR Assay

To assess the percentage of stem like cells in culture, an ALDEFLUOR assay was ran on H460 cells and H460 spheroids. The ALDEFLUOR measures aldehyde dehydrogenase (ALDH) activity within the cell by generating a fluorescent product, effectively enabling measurement via a flow cytometer. ALDH expressing cells are associated with a stem like nature. For the H460 cells, the plates were trypsinized for 5 minutes and inactivated with media. After quantitation with a hemocytometer, 200,000 cells were aliquoted from the cell suspension and placed into a 15 milliliter conical tube and centrifuged at 800 RPM for 5 minutes. Supernatant was aspirated away without disturbing the cell pellet. The cells were washed with 10 mL of dPBS and centrifuged at 800 RPM for 5 minutes. The supernatant was then aspirated off and the pellet was resuspended into 1 mL of the ALDEFLUOR assay buffer. Two 4 mL test tubes were labeled as experimental and control. The 1 mL of cell suspension resuspended in the assay buffer was then transferred into the experimental tube. Five uL of the DEAB (ALDH inhibitor) solution was pipetted into the control tube. Five uL of the activated ALDEFLUOR substrate was pipetted into the test tube and immediately afterwards 500 uL from the test tube was transferred into the control tube. Samples were incubated for 40 minutes at 37° C. The tubes were then centrifuged at 250 x g for 5 minutes and the supernatant was aspirated. Lastly, 500 uL of ALDEFLUOR assay buffer was added to both tubes and stored on ice. The samples were ran on a cytometer as soon as possible, as the fluorescence drops in a time dependent manner.

Folate-FITC Assay

To quantitate the amount of available folate receptors on the H460 cells FACS analysis was done using folate conjugated to FITC. H460 cells were grown in folate-free media for 2 weeks prior to testing. This was done to permit folate receptors to reach a level more akin to what it would be *in vivo*. Controls for this experiment were acquired by using KB cells, which are known to have high cell surface folate receptor levels. To avoid degradation of any surface receptors, cells were trypsinized for no longer than 5 minutes. Trypsin was then inactivated using folate-free media and cells counted. After quantitation, 100,000 cells were aliquoted out of suspension and centrifuged at 800 RPM for 5 minutes. Supernatant was aspirated and 500 uL of minus folate and minus serum media was dispensed into the positive control and the experimental tube. For the negative control, dispense 500 uL of plus folate minus serum media, which will compete with the folate-FITC conjugate. Afterwards, 50 uL of the folate-FITC conjugate was pipetted into each suspension and incubated for 30 minutes. Suspension was then washed twice with cold dPBS and centrifuged at 800 RPM for 5 minutes. Supernatant was aspirated and replaced with 500 uL of dPBS. Samples are ready to be ran on a cytometer at this point.

SDS PAGE Western Blotting

Cells were plated on 40 mm dishes at 100,000 per plate and let set for ~24 hours. Desired verteporfin and reducing agent concentrations were made and added to dishes. After 24 hours, media was aspirated and washed with ice cold PBS. Cells were lysed in 200-300 uLs of phospho-tyrosine lysis buffer (50 mM Hepes, pH 8.0, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4 , 10 mM NaF, 10 mM NaH_2PO_4 , 100 KIU/ml aprotinin and 1 mM PMSF). Plates were scraped and re-aliquoted into sterile micro-centrifuge tubes. Lysates were centrifuged at 800 G for 10 minutes at 4° C. Supernatant was added to a fresh micro-centrifuge tube with the pellet being discarded. The Bradford protein assay was conducted to acquire protein concentration per lysate. Using the given concentrations, thirty micrograms of protein was pipetted from lysates and added to ten microliters of 4x loading buffer. Samples were placed into a water bath set at 90° C for five minutes to denature sample proteins and coat them with sodium dodecyl sulfate. After denaturation, samples were placed in a micro-centrifuge to pull down any water that was evaporating from the boiling process. The entirety of the samples were then pipetted into individual wells in an 8 % poly-acrylamide gel. BD bioscience all-blue protein ladders were used to delineate protein size. Gels were ran with ice cold running buffer at 90-100 milliamps for 90 minutes. Once the run was completed, the transfer apparatus was prepared. PVDF membranes were activated in 100% methanol and placed on top of two filter papers soaked in transfer buffer. The gel was taken out of the running apparatus and placed on top of the membrane, assuring no formation of air bubbles. Another filter paper soaked with transfer buffer was placed over the gel. A roller was used to force any air bubbles that may have formed out of the compilation. The

clamp was closed and submerged into the transfer apparatus. A constant voltage was used, set at 100V, to transfer the proteins set on the gel to the PVDF membrane. The transfer was ran for one hour with an ice pack placed inside. After completion a Ponceau Stain was conducted to confirm proper transfer of proteins. At this point, the membrane was blocked for 24 hours in 5% skim milk at 4° C. After blocking, the membrane was washed twice in tris-buffered saline with 1% Tween-20 added (TBST). Membrane was split in half, cut underneath the 50 kilodalton band on the protein ladder.

PRIMARY ANTIBODY	Antibody dilution inTBST (uL)
P62	1/5000
YAP	1/2000
LC3	1/3000
β -Actin	1/10000
GAPDH	1/10000

Table 1. List of antibodies and dilutions.

Primary antibodies were diluted, as listed above, and added to the respective membrane for one hour. Three washes with TBST for 5 minutes were conducted to wash off any nonspecific binding. After washing, the secondary antibody was added to both membranes for thirty minutes (Anti-Mouse HRP 1:30000 TBST). Another series of washes was conducted, a total of five for five minutes per wash with TBST. Using enhanced chemiluminescence, the membranes were developed in a dark room using autoradiography.

Reverse Transcriptase PCR

Cells were set at 250,000/10 cm dish dish and placed into a cell culture incubator for 24 hours. Media was aspirated and replaced with fresh media infused with verteporfin concentration of choice for 24 hours in a cell culture incubator. After the 24 hour exposure, media was aspirated off, and the dish was washed with ice cold PBS and aspirated off. One milliliter of Tri-Reagent was placed into each dish. Using a sterile scraper, the entire area of the dish was scraped and the Tri-Reagent was pooled into an area on the dish. The entirety of the solution was then pipetted into a sterile DNAase and RNAase free micro-centrifuge tube. At this point, tubes were either frozen at -80° C being processed at a later time or RNA was immediately isolated out of the solution. When isolating RNA, 200 uL of chloroform was pipetted into each micro-centrifuge tube and inverted for at least 15 seconds. After 5 minutes, at room temperature, tubes were centrifuged at 12,000 G for 15 minutes at 4° C. The tubes were then carefully placed into a clean rack and the clear supernatant was pipetted into a fresh DNAase and RNAase free micro-centrifuge tube. To precipitate out the RNA, 500 uL of isopropanol was then added. After 5 minutes, the tubes were centrifuged at 12,000 G for 10 minutes at 4° C. The supernatant in the tubes is discarded and then washed with one milliliter of 70% ethanol. The tubes were centrifuged at 12,000 G for 5 minutes. Supernatant is discarded without disturbing the pellet. The tubes are then incubated in a water bath at 37° C until the remainder of the ethanol has evaporated from the tubes. The RNA isolated in the pellet was then resuspended in 15 microliters of RNAse and DNase free water. The tubes were then incubated at 50° C for 5 minutes to fully dissolve the RNA into solution. The concentration of RNA was then quantitated using a Nanodrop spectrophotometer. At this

point samples were standardized for reverse transcription, aliquoting 5 ug of RNA into PCR grade RNase and DNase free micro centrifuge tubes. Samples were then QS to 18 microliters. In a separate container, random hexamers and dNTPs were mixed; using 4 microliters of random hexamers per sample and 2 microliters of 10 mM dNTP per sample. Six microliters of the random hexamer and dNTP solution was dispensed into each sample for a total of 24 uL per tube. To facilitate annealing, micro centrifuge tubes were placed into a 60 degree water bath for 5 minutes. During this incubation the reverse transcriptase master mix was prepared. The master mix was comprised of 9 uL of nuclease free water, 1 uL of RNASE OUT, 4 uL of 10x reverse transcriptase buffer, and 2 uL of MuLV reverse transcriptase. After the 5 minute incubation, the samples were taken out of the water bath and wiped dry with a Kim wipe. Afterwards, 16 uL of the reverse transcriptase master mix was dispensed into each sample tube. The samples were then incubated at 50° C for 50 minutes, and then 70° C for 5 minutes to denature the RT enzyme. The cDNA product was measured on the Nanodrop on the single strand DNA setting to acquire approximate cDNA values. At this point samples were standardized so that each contained the same amount of cDNA, assuring that enough diluent is used to perform the reactions desired. The master mix was assembled next; which consisted of 3 ul of nuclease free water, 1 ul of both the forward and reverse primers, and 10 ul of 2x syBR green mix. With the mix completed, 15 ul was pipetted into a 96 well PCR grade plate followed by 5 ul of the diluted cDNA sample. The plate was centrifuged and placed onto an Eppendorf Mastercycler to begin and measure the reactions.

RESULTS

Preface

The basis for this work is that verteporfin has been shown to block aberrant YAP activity, and since NSCLC has been shown to be correlated with abnormal YAP activity (5,16-18), it can be hypothesized that proliferation and survival may decrease substantially by treatment with verteporfin. Beyond proliferation and survival, the change in gene expression brought on by verteporfin may sensitize stem-like populations by the change in gene expression. If true, verteporfin would be a great candidate for synergistic drug therapies as the stem cell populations of tumors are more resistant to traditional treatments. Another facet of verteporfin treatment that will be touched on is the oligomerization of p62 and the disruption of autophagy. Disrupting autophagy would have detrimental effects on tumor cells. This would put stressors on the cells that could lead to apoptosis or impaired function. All of these qualities may make verteporfin a very powerful tool in the fight against NSCLC.

The only problem that can be gleaned at this point is that there are no specific delivery systems to the cancer cells. In cell culture this is not an issue, but at the scale of an organism treatment may be difficult. A method that was looked into to aid in specificity was the conjugation of folate onto verteporfin. Tumor cells typically have upregulated folate receptors, as they are highly proliferative. This can be used to deliver verteporfin more specifically to them. This will be touched on in the last section of the results.

To start off, proliferation and survival are looked at in H460 cells and H23 NSCLC cells with the treatment of verteporfin.

Verteporfin Reduces Proliferation and Survival of NSCLC Cells

To assess how treatment with verteporfin would affect NSCLC cell viability, an MTT assay was conducted. Several pilot experiments performed to ascertain how long the drug should be left on to have noticeable effects. The exposure time varied from 72 hours, 96 hours, to 120 hours for these pilot experiments. The difference in effectiveness was negligible, therefore 72 hours was chosen as the exposure time for the subsequent experiments.

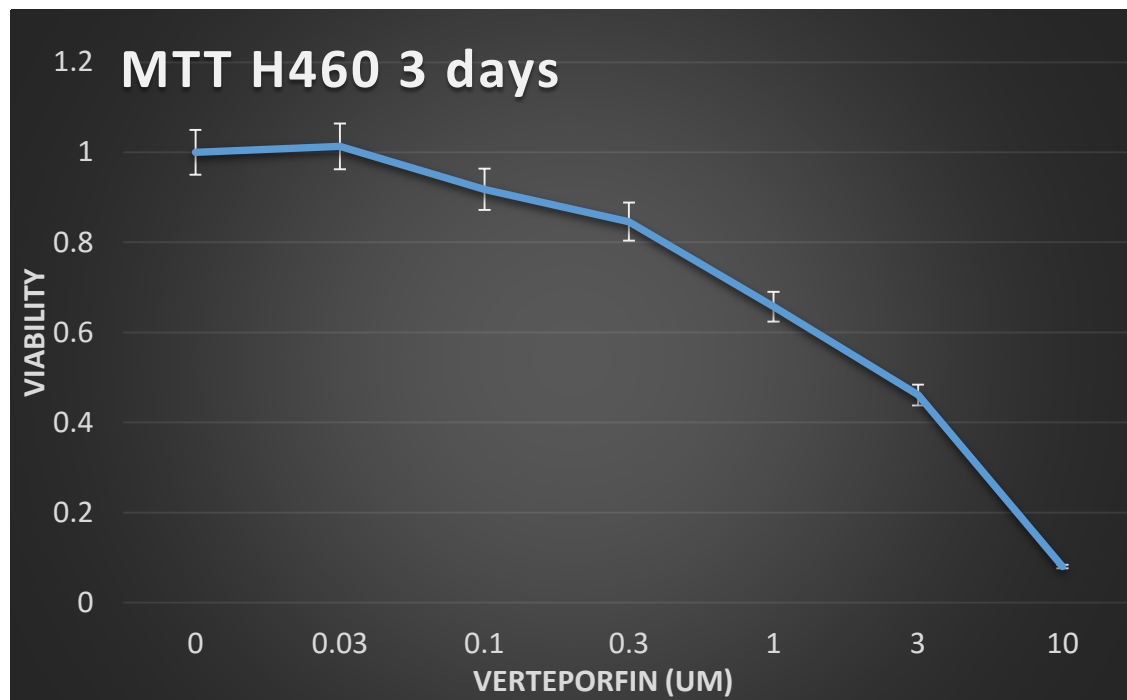
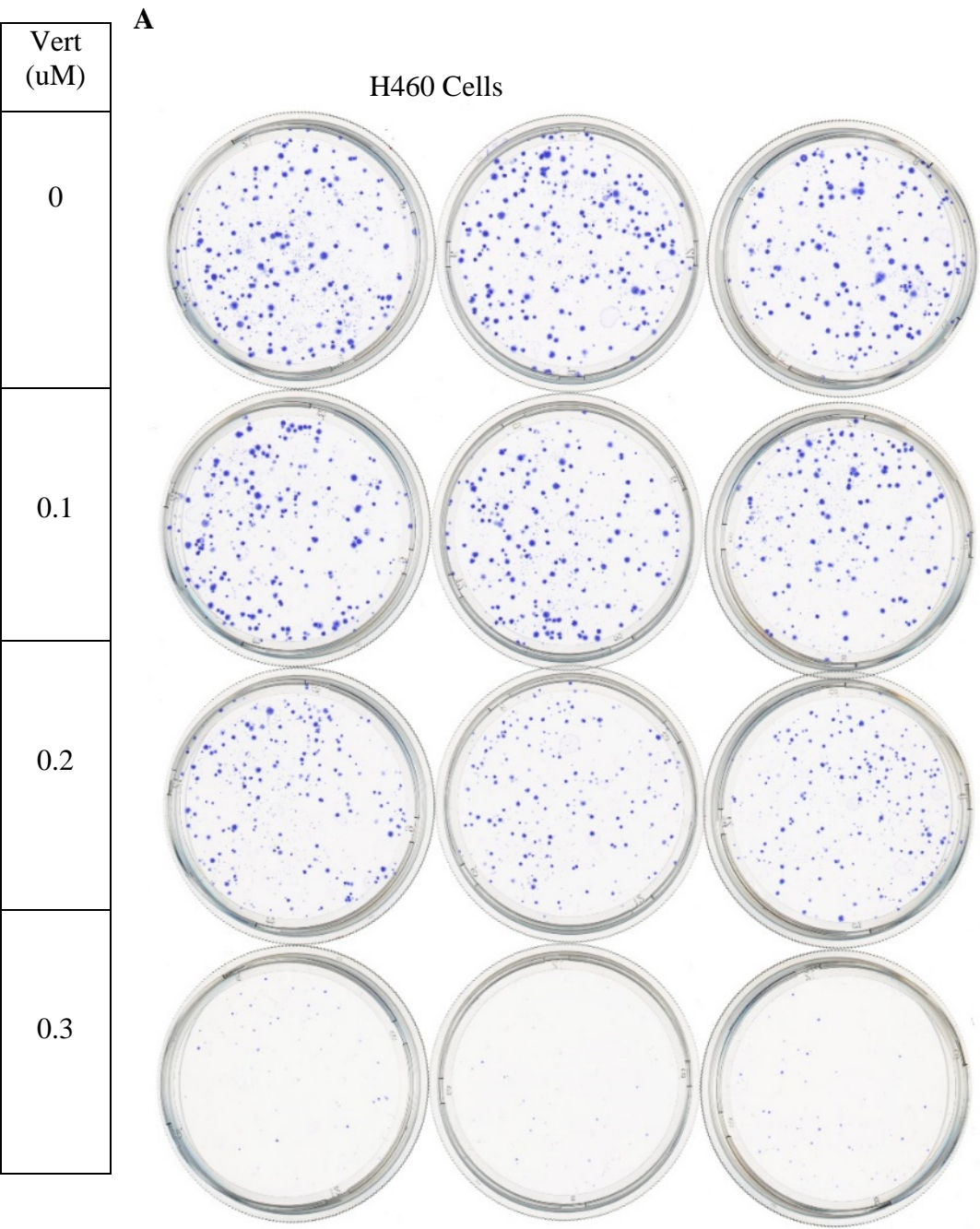


Figure 3. Verteporfin dose-dependently inhibits NCI-H460 cell viability. Viability is standardized to the control plate. cells were incubated with the indicated concentrations of verteporfin or DMSO solvent alone (all points were adjusted to 0.1% DMSO) for 72 hours prior to performing the MTT assay. Data points were collected in triplicates. The IC50 is approximately 2.75 uM.

Results from the MTT assay show that a negative correlation between the concentration of verteporfin and NSCLC cell survival exists. To further reinforce that viability is decreasing as well as to get a more accurate IC₅₀, a series of clonogenic survival assays were conducted. Approximately 500 cells were seeded onto 60 mm dishes a day before their exposure to verteporfin. Initial experiments established a narrower effective concentration of verteporfin than in the MTT assay. Therefore doses between 100 and 500 nM were selected for subsequent survival assays. Dishes exposed to verteporfin are shown below in figure 2 for both the H460 and H23 cell line for three days.



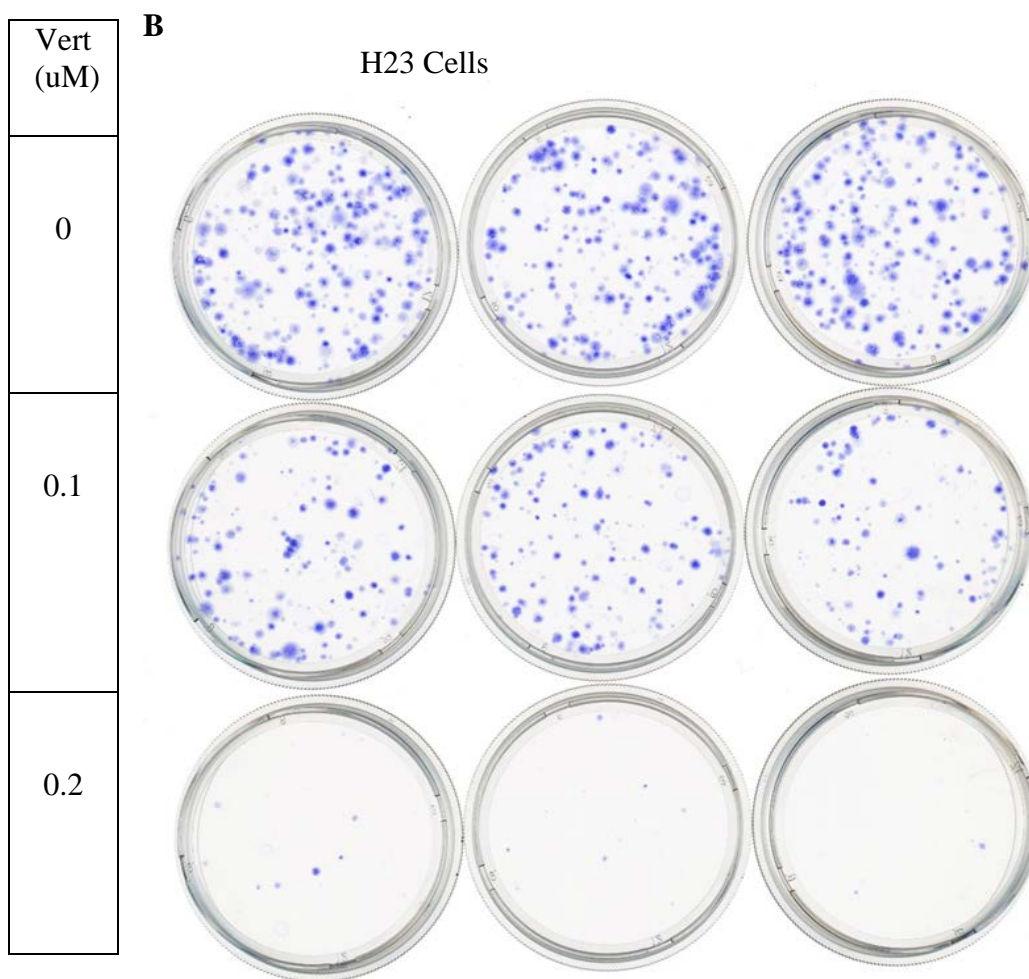


Figure 4-A. Verteporfin reduces survival and proliferation in H460 NSCLC cells.

Cells were exposed to DMSO solvent or verteporfin in a dose dependent manner for 72 hours. After exposure, cells were washed and allowed to recover for 7-9 days. Cells were then fixed and stained with crystal violet for colony visualization. The IC₅₀ for H460 cells is approximately 0.2 uM of verteporfin.

Figure 4-B. Verteporfin reduces survival and proliferation in H23 NSCLC cells.

The IC₅₀ in the H23 cells is 0.1 uM of verteporfin. Fewer colonies demonstrates that survival is decreasing. Smaller colonies is indicative of decreased proliferation. Higher concentrations that were used (resulting in complete loss of colony survival) are not shown.

Surprisingly the IC₅₀ was considerably lower in the clonogenic assay than it was in the MTT assay. This unexpected result yields some insight into how verteporfin may be reducing viability. There are several plausible mechanisms of actions for this result.

Cells are much more dispersed and in less dense colony formations in the clonogenic assay compared to the MTT assay. This would make the cells initially more dependent on Hippo signaling, since the cells had their junctions severed during the trypsinizing process and weren't able to cluster back together. In the MTT assay the surface area is greatly reduced, allowing the NSCLC cells to cluster and perhaps be less dependent on the Hippo pathway. Increased density may be one reason why verteporfin is much more effective in the clonogenic assay as cells that are more dependent on the Hippo pathway may be more sensitive to verteporfin treatment. Another mechanism may just be a matter of time. In the clonogenic assay, after 3 days of exposure to verteporfin the media is aspirated, the plate is washed and replaced with fresh media; the cells are left to recover for several days at this point. This recovery period may facilitate the cells transition to an apoptotic state (significant cell death observed at the higher drug doses was not typically observed until after the cells had been in culture for 4-5 days). The last mechanism that was thought of is the potential for verteporfin to remain in the cytosol of the NSCLC cells after the plates were washed. Since verteporfin is hydrophobic, it should flux in and out of a cell with relative ease. However, if for some reason it gets sequestered in the cytosol the recovery period may just be elongating exposure time.

With these two assays it can be said with a degree of certainty that verteporfin does indeed reduce the proliferative capacity as well as the survivability of NSCLC cells. The next step was to figure out how this might be accomplished.

Blocking the YAP-TEAD Interaction

The basis for verteporfin to be effective in reducing the viability of NSCLC was believed to be its blocking of YAP from binding to the TEAD family of transcription factors. To confirm that verteporfin is attenuating YAP activity in NSCLC cells, a series of RT-PCR assays were performed. The gene CTGF was chosen to measure the efficacy of verteporfin to downregulate TEAD responsive genes, as CTGF is a prominent gene associated with YAP-TEAD activity and the most frequently monitored (19,20).

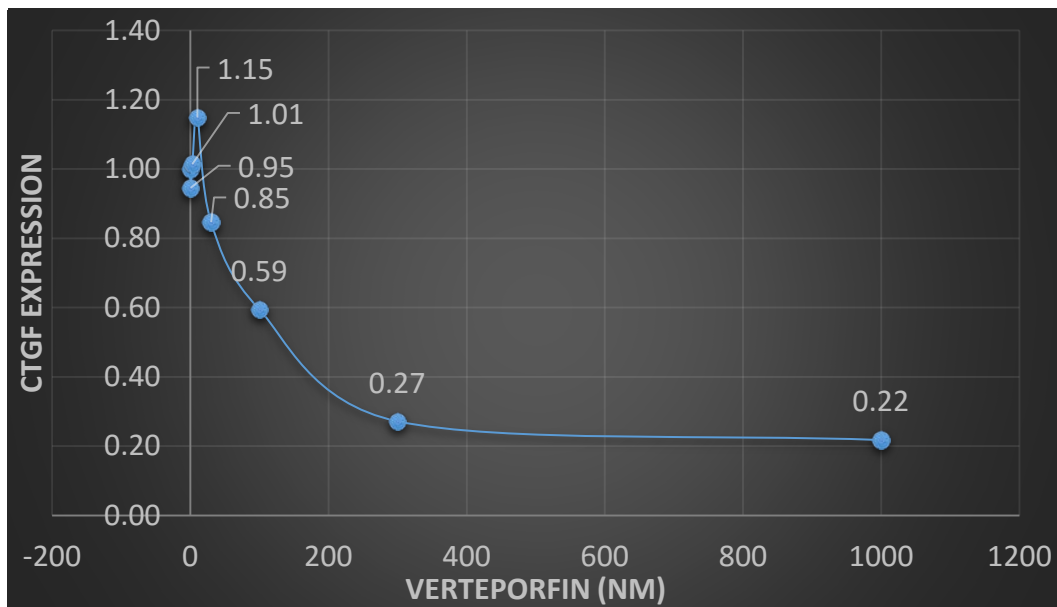


Figure 5. CTGF expression is markedly reduced with the addition of verteporfin to media. H460 NSCLC cells were exposed to verteporfin for 24 hours at the concentrations above. Calculations were done using the comparative Ct method. This figure was generated by a member of the Quilliam lab.

As expected, verteporfin decreases the expression of CTGF mRNA in a dose dependent manner. The IC₅₀ was just under 200 nM and by 300 nM CTGF expression is

nearly a quarter what the control plate expressed, this shows how potent verteporfin is in inhibiting downstream TEAD transcription. This is a massive decrease that could potentially be extended to say that genes associated with TEADS are decreased perhaps to the same extent; phenotypically this means proliferation, survival, and a stem like nature should decrease as well. To confirm the above statement, other downstream TEAD genes would have to be monitored with verteporfin treatment. The results of the PCR correlate nearly perfectly with the dose response curve for the clonogenic assay. Which suggests that the mechanism of death for these cells may be through the Hippo pathway.

A contending hypothesis against the above role of verteporfin acting through inhibition of YAP-TEAD signaling was described shortly after my project began; verteporfin was shown to promote the oligomerization of p62, which blocks autophagy and may lead to cytotoxic apoptosis. The next section of the results will touch on this aspect of NSCLC cell response to verteporfin treatment.

Low Concentrations of Verteporfin Covalently Homo-Oligomerize p62

Donohue et al showed that verteporfin covalently homo-oligomerizes p62 and inhibits autophagy at 10 uM without light activation (10). However, they do not show that that this crosslinking happens with the low concentrations used in the figures 4 and 5 herein. An earlier study by this group also suggested that verteporfin impacted autophagy, but again, the doses of drug used were quite high compared to the nM levels used in figures 4 and 5. Initially, it was assumed that these oligomeric p62 constructs would not with the low concentrations of verteporfin being used in my studies. However, results proved these assumptions wrong. Numerous western blots were conducted, probing for p62, at different time points and concentrations demonstrating this finding. In subsequent experiments, antioxidants were used to try and prevent p62 oligomers from forming. Preventing p62 from oligomerizing would help establish if verteporfin is acting through the Hippo pathway or by other means. The antioxidants used were glutathione, α -tocopherol, n-acetyl cysteine, and histidine. Histidine was shown to be the most effective at preventing p62 oligomers from forming and was used hereafter as a singlet oxygen squelcher.

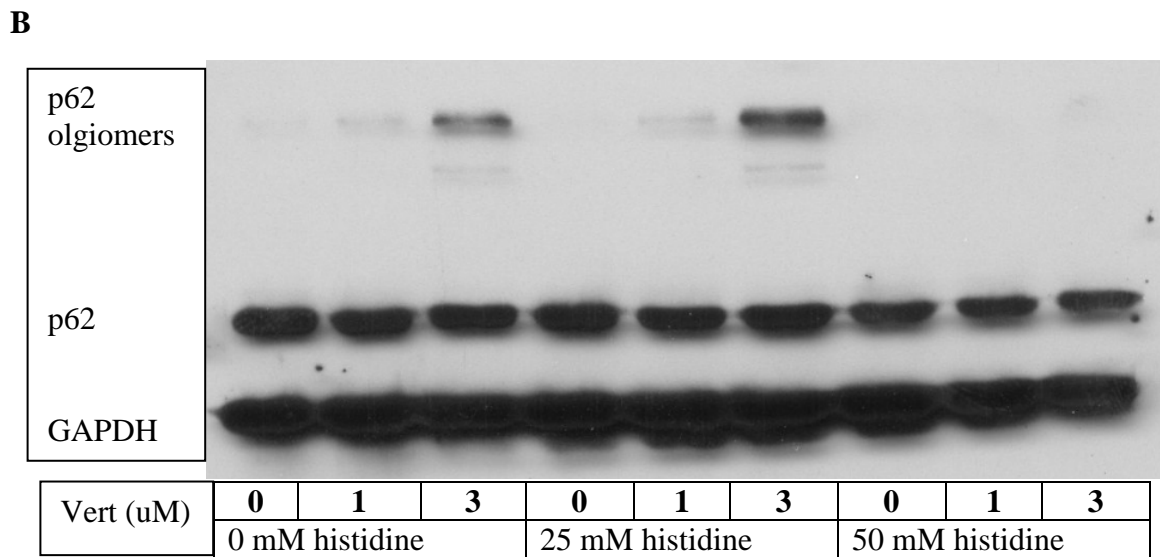
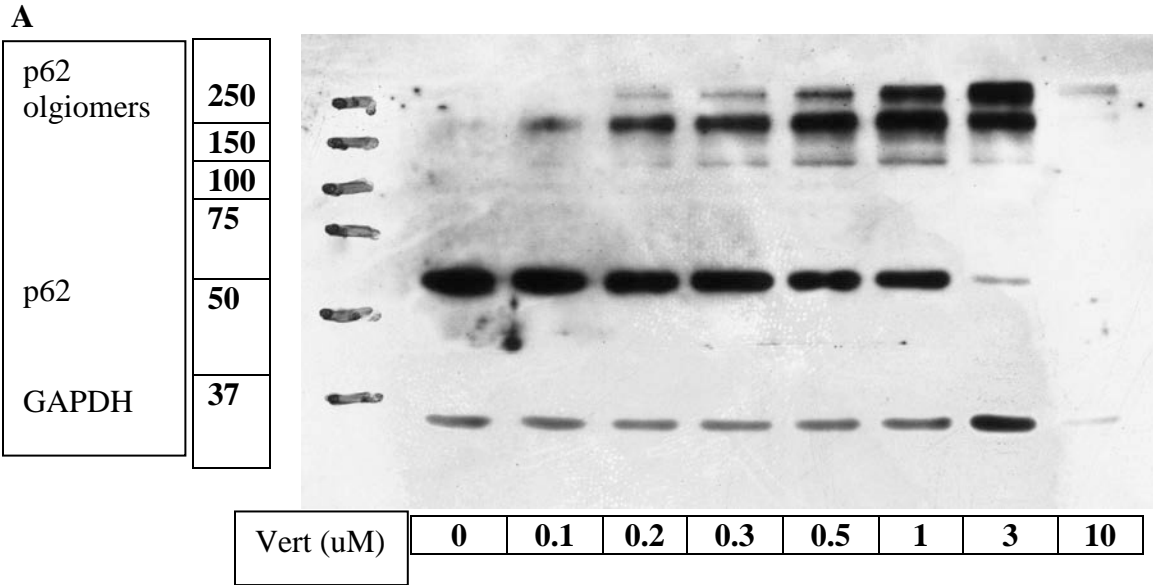


Figure 6-A. Dose-dependent induction of H460 cell p62 oligomerization by verteporfin under low light conditions. Cells were exposed to indicated levels of verteporfin for 24 hours. Size markers are indicated in kDa

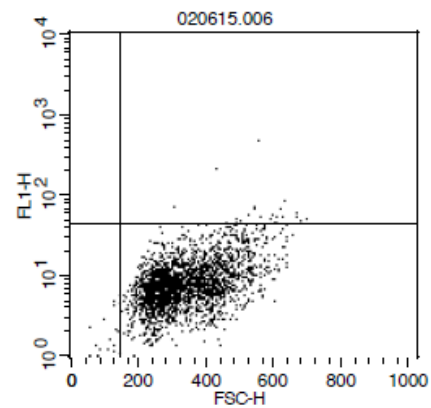
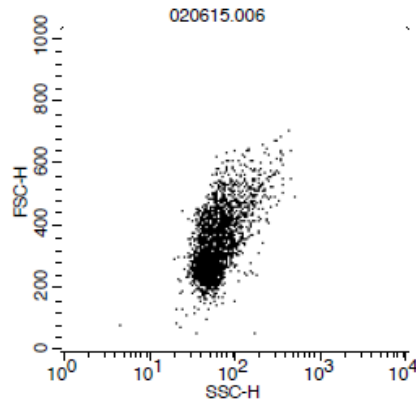
Figure 6-B. Impact of histidine on verteporfin-induced p62 oligomerization. Cells were exposed to compounds for 12 hours. Histidine was shown to prevent p62 oligomerization at 50 mM.

As shown above in figure 4-A, verteporfin causes high molecular weight p62 bands to appear as the dosage increases. This was expected, but not for the 100-200 nM range. Also, the exposure time is one-third that of the MTT and clonogenic assays. This is problematic for the initial hypothesis, as this puts a hole into the prior thinking. However, it is important to note that the samples used in figure 4-A were exposed to light during the processing of the samples, which may have had an effect on oligomerization. To discern whether oligomerization can be prevented or reduced, histidine was used as a singlet oxygen squelcher. As shown in figure 6-B, 50 mM of histidine blocks the oligomerization of p62 for 1 and 3 μ M of verteporfin exposed to the cells for 12 hours. The MTT, clonogenic, and RT-PCR need to be redone using histidine in conjunction with verteporfin, to test if the previous results are attributed to the blocking TEAD downstream genes or cytotoxicity. Due to some unfortunate circumstances (several stock preparations of verteporfin lost effectiveness), it has been problematic to follow through with these experiments.

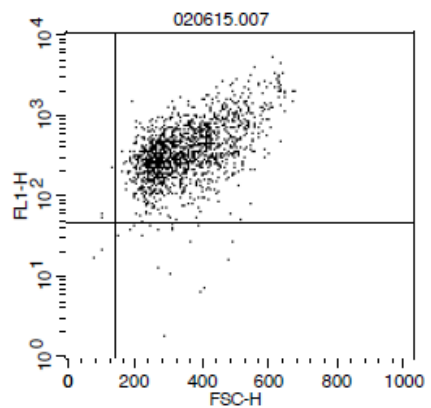
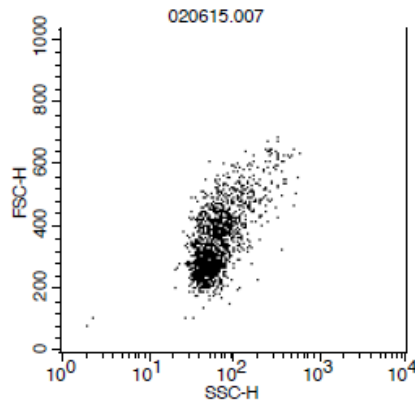
Folate-Verteporfin Conjugate

The conjugation of folate to verteporfin could potentially make it more specific for proliferating cells. Previous studies have shown the efficacy of conjugating folate to molecules to enhance delivery (21). This would be a major benefit in making verteporfin into a viable systemic treatment option. Another added benefit of folate conjugation might be increased solubility, which would further improve drug delivery *in vivo*. To test this, a preliminary study was conducted to quantify the level of folate receptors present on the NSCLC cell line H460. Quantifying the number of folate receptors is key, as they're anticipated to be the major conduit of the conjugate into the cells. Once inside the cell, the folate should be cleaved off, which leaves verteporfin's activities unchanged. However, if there are not enough folate receptors present, then the driving force will be diminished. NSCLC is known to have high levels of folate receptors (22), but conditions may change in cell culture. Cells have been passaged and grown in RPMI, which has a very high level of folate which would promote endocytosis and down-regulation of receptors. Before testing the amount of folate receptors, the cells were passaged in folate free media for two weeks. This was done in hopes of restoring the folate receptors back to *in vivo* conditions.

**KB cells
negative
control**



**KB cells
positive
control**



H460

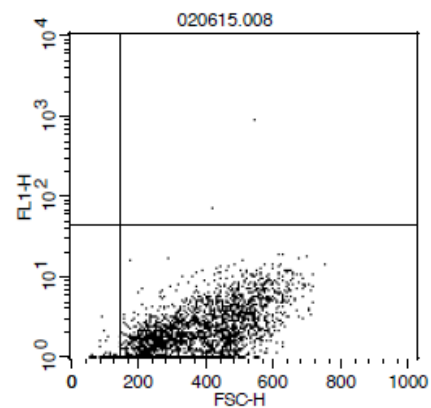
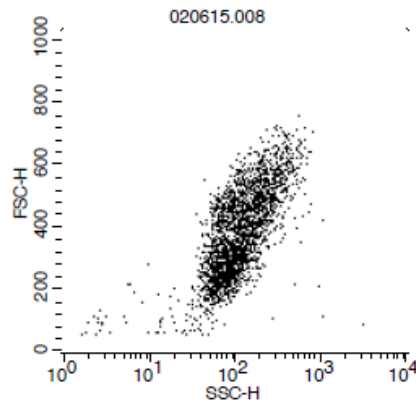


Figure 7. H460 cell line has a relatively low level of folate receptors.

This experiment shows the relative amount of folate receptors present on the cell membrane. The first two plots are control runs with KB cells, which are known to be highly positive for folate receptors. This facilitates a negative and a positive control, with and without folate competition, respectively. The third chart depicts the relative amount of folate receptors on the H460 cell line after being cultured in the absence of folate for ~10 days.

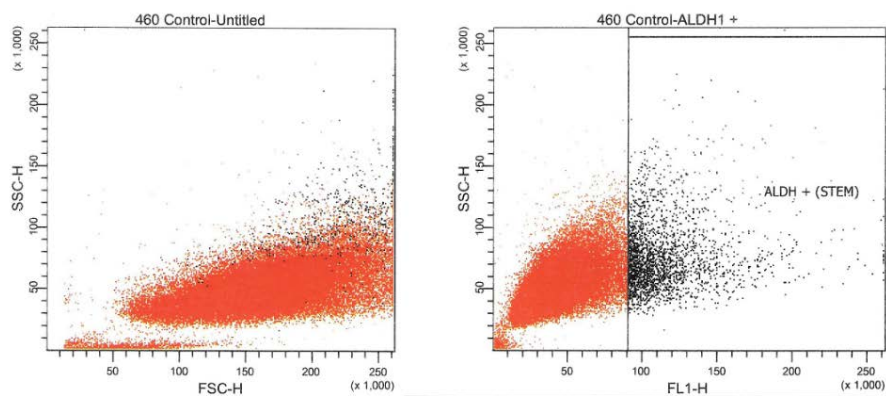
The data from the folate uptake assay shows that there are very few folate receptors on the surface of the H460 cell line compared to the positive control, KB cells. This is unfortunate, as this cell line wouldn't apply for testing the verteporfin-folate conjugate. This conjugate may prove to be a very effective delivery model *in vivo*, but in cell culture, it may not apply very well, unless the appropriate cell line was selected. The KB cell line could be suitable for pilot experiments until an appropriate NSCLC cell line is identified.

H460 NSCLC Cultures Have a High Percentage of Stem Cells

Typically, stem cells are resistant to traditional chemotherapeutics, which are thought to transport the drugs outside of the cell before they have any effect. Verteporfin being hydrophobic, can flux back into the cell even after being transported out. More importantly, TEAD-induced signaling supports stem cell maintenance. So, verteporfin could potentially thwart a stem cell phenotype, possibly making them less resilient. To measure the percentage of stem cells and to test if verteporfin may be more selective for stem cells, an ALDEFLUOR assay was piloted, since stem cells express high levels of aldehyde dehydrogenase isoform 1A1 (ALDH1A1) (23). The rationale behind this being that stem cells are more reliant on the Hippo pathway for survival (24). If this is the case, verteporfin could be quite effective at limiting their numbers. The ALDEFLUOR assay, combined with Flow Cytometry, measures ALDH activity within the individual cells, which is a reliable marker for stemness (25). The assay was conducted on H460 cells and H460 spheroids, the latter being enriched with stem cells since they are resistant to anoikis.

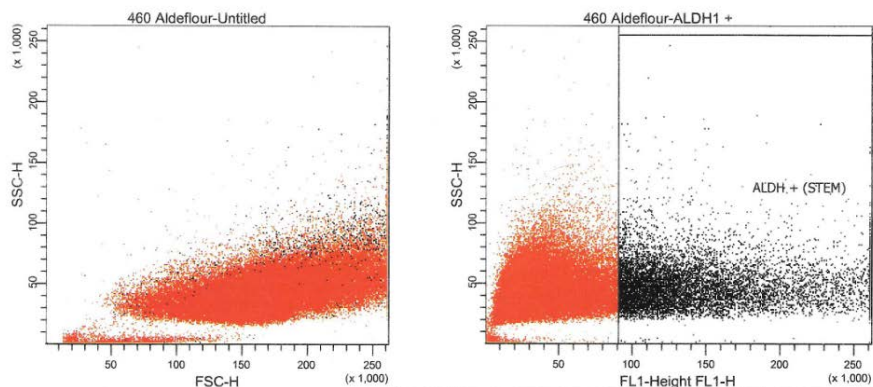
The results of the assays showed that after subtracting the isotype from the experiment, the H460 cells have a 5.7% ALDH-positive stem population while the H460 spheroids have 17.7%. This is a considerable stem population, which could yield good results for the concluding experiments. Unfortunately, my work does not include the next step in the process, which would be to add verteporfin at varying concentrations and see if the stem populations lessen with higher concentrations. The results are shown below.

A



Experiment Name: Experiment_004
 Specimen Name: 460 Control
 Tube Name: Untitled
 Record Date: Feb 20, 2015 12:42:04 PM
 GUID:
 \$OP: flowlab

Population	#Events	%Parent	%Total	SSC-H Mean	FL1-H Mean
■ H460	66,390	####	100.0	49,242	40,837
⊠ ALDH + (STEM)	2,369	3.6	3.6	76,699	116,484
■ ALDH -	64,021	96.4	96.4	48,226	38,037



Experiment Name: Experiment_004
 Specimen Name: 460 Aldeflour
 Tube Name: Untitled
 Record Date: Feb 20, 2015 12:45:45 PM
 GUID:
 \$OP: flowlab

Population	#Events	%Parent	%Total	SSC-H Mean	FL1-Height FL1-H Mean
■ H460	100,000	####	100.0	40,184	41,096
⊠ ALDH + (STEM)	9,281	9.3	9.3	47,927	149,866
■ ALDH -	90,719	90.7	90.7	39,392	29,969

B

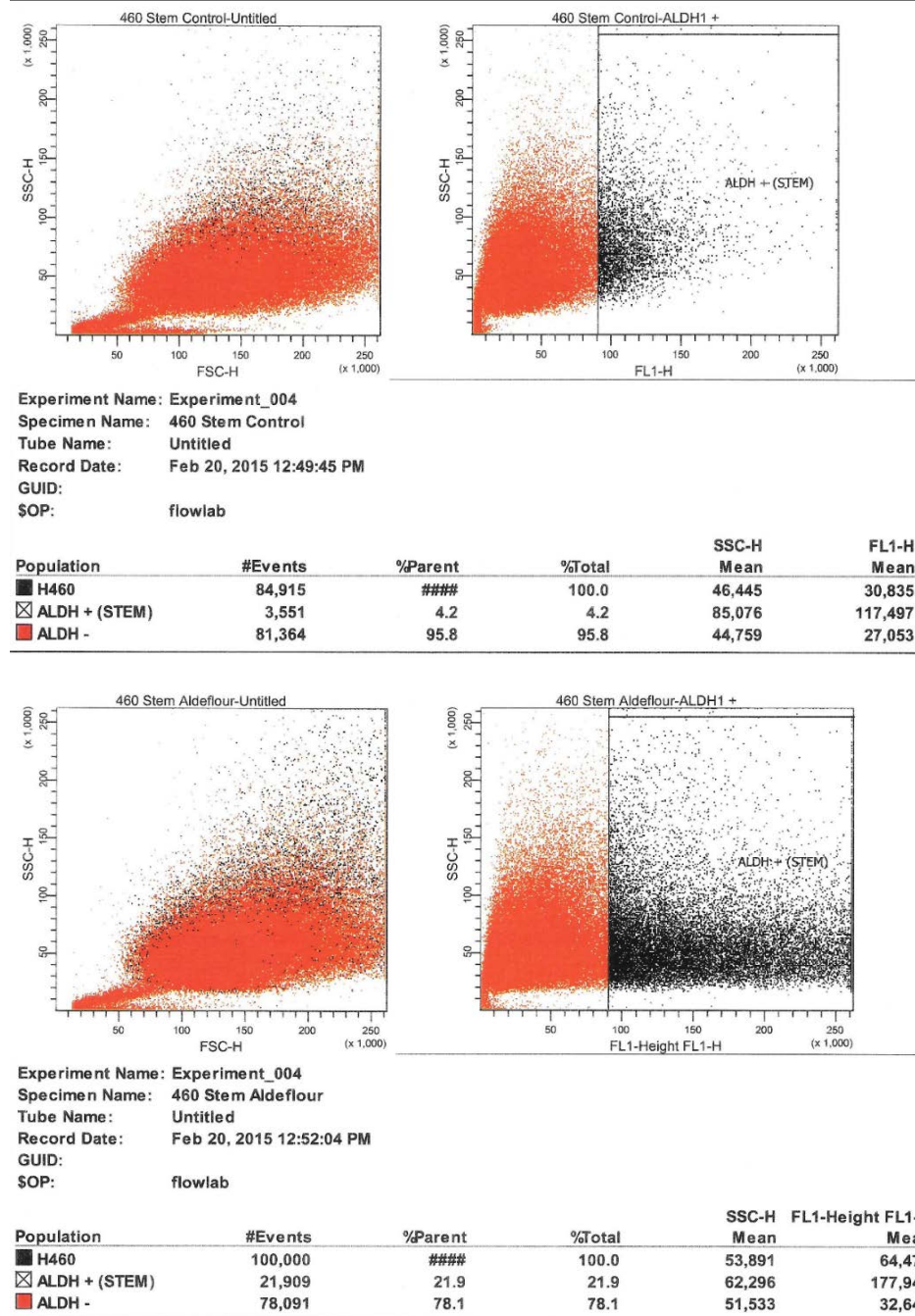


Figure 8-A. H460 NSCLC cultured have a relatively high percentage of stem cells. The first run is a control that allows an accurate placement of the gate, which transfers over onto the experimental run. H460 cells were shown to have a 5.7% stem population. **Figure 8-B. H460 NSCLC spheroids have a 17.7% stem cell population.**

DISCUSSION

Preface

With the conclusion of the results section, I am going to talk about the future avenues that this work could pursue. In my work, it is established that verteporfin affects NSCLC cell viability. However, there are many areas left blank that this work can be a preamble to. For instance, developing and/or acquiring a mouse model for NSCLC would help fortify if verteporfin would be affective *in vivo* or not. This among other possible experiments will be discussed in the future experiments subsection.

Prior to going over future experiments, ideas and theories will be deliberated about verteporfin and the phenomenon that was seen in my experiments with NSCLC. There have been many studies with verteporfin being used as a therapeutic agent to treat a variety of mouse models of cancers before and after my work began. Some of these studies will be discussed to attempt to form a more complete picture.

Ideas and Interpretations

In my work with NSCLC it is seen that the TEAD-responsive gene CTGF is downregulated and p62 is covalently homo-oligomerized with the treatment of verteporfin *in vitro*. Therefore, verteporfin is likely to have at least 2 potential mechanisms of action as supported by the literature: (i) inhibition of YAP/TEAD-induced gene expression that is associated with survival and proliferation and (ii) protein aggregation resulting in disruption of the processes such as autophagy. One or both of these mechanisms is responsible for the affects seen in viability, discerning what mechanism is responsible for the decrease in viability was an unfinished aim for my study. This unfinished aim is important, however, the key point is that verteporfin effectively reduces/kills tumors and is not toxic to normal tissues. Studies by others have been conducted showing verteporfin's efficacy in reducing tumor size while not being cytotoxic to normal tissue *in vivo* (9,11). The main modality for cell death differs for the two studies referenced above. Liu-Chittenden et al reports that verteporfin suppresses liver overgrowth and the formation of hepatocellular carcinoma through the perturbation of the YAP-TEAD interaction. While Zhang et al reports that YAP-TEAD has nothing to do with reducing colon cancer size or proliferation and that the effects are strictly through proteotoxic means. This difference in mechanism is very interesting. It may mean that verteporfin might promote apoptosis through different means, depending on what the cancers weak points are. As long as there are no deleterious effects on normal tissue; which the two studies above show very little toxicity in normal tissue, verteporfin could be a great drug for metastatic cancers. The duality of blocking the YAP-TEAD interaction and promoting protein crosslinking through its singlet oxygen production are

very powerful modes of limiting proliferation, particularly if the crosslinking inhibits autophagy.

Blocking the YAP-TEAD interaction leads to a decrease in transcription in genes associated with proliferation, survival, and stemness. In other words, blocking the YAP-TEAD interaction decreases proliferation and sensitizes cells to apoptosis while decreasing a stem-like nature. These qualities likely make verteporfin synergistic with other compounds. Appropriate drugs to combine with verteporfin may be cisplatin or erlotinib. Clonogenic and MTT pilot assays were conducted with cisplatin as a candidate for synergism, used during, before and after verteporfin treatment. There were no discernable differences between the effectiveness of the combination. However, these experiments were done while verteporfin was beginning to lose its potency in my assays, so the results were not conclusive and not included herein. However, studies by others in the lab indicated that verteporfin cooperates with erlotinib in a mutant EGF receptor NSCLC cell line, HCC4006, to promote cell death. In addition, after all my experiments were concluded, a study reported that ablating YAP1 improves sensitivities to other modes of treatment in NSCLC (26). This fortifies the thinking that verteporfin would be effective at synergistic treatments and should be further studied. Since the maximal effects of verteporfin on inhibiting cell survival and CTGF expression occurred at lower doses than the induction of p62 oligomerization, it is possible that cell death in my hands was more dependent on YAP-TEAD signaling.

Many other proteins other than p62 are more likely than not crosslinked during treatment with verteporfin; ones that have been confirmed are STAT3 and lamins (11). Also, PCNA maybe crosslinked during treatment, since a study has reported that it is

crosslinked with the introduction of singlet oxygen causing agents (27). If the cells have normal autophagic and/or proteosomal activity these may be able to be dealt with. Cells under stressors, such as tumor cells, may be unable to deal with the added burden of compromised proteins building up in the cytosol. Of the proteins that are known to crosslink, it is known that the functioning of autophagy and mitosis may be compromised (10,27). This is what I believe to be the most likely cause of the decrease in viability. While YAP-TEAD genes are down-regulated during verteporfin treatment, creating a higher propensity to become apoptotic, non-functional protein oligomers build up ultimately leading to the cell's failure. Zhang et al came to this conclusion in his studies with the use of verteporfin in the treatment of pancreatic cancers (11). I believe the same to be true for NSCLC. Further study is needed in different tumor types to get a more definitive answer.

The following is an experiment that I did not include within the results sections because of the inconsistency of the results. The inconsistencies of the results were assumed to be from verteporfin having degraded in the stock solution. However, after using fresh verteporfin the results were still inconsistent, which leads me to believe there were issues elsewhere. It was noted that FBS lots were changed around the month results became erratic, but unfortunately, the problem was never resolved.

Several RT-PCR's were ran with the H460 cells being exposed to histidine and verteporfin at the same time. In the experiment shown below, in figure 9, verteporfin is proficient in down regulating CTGF in the presence of 25 mM of histidine. This suggests that verteporfin is not acting through singlet oxygen as far as down regulating TEAD-

responsive genes. This experiment would have to be repeated several times to get a conclusive answer though.

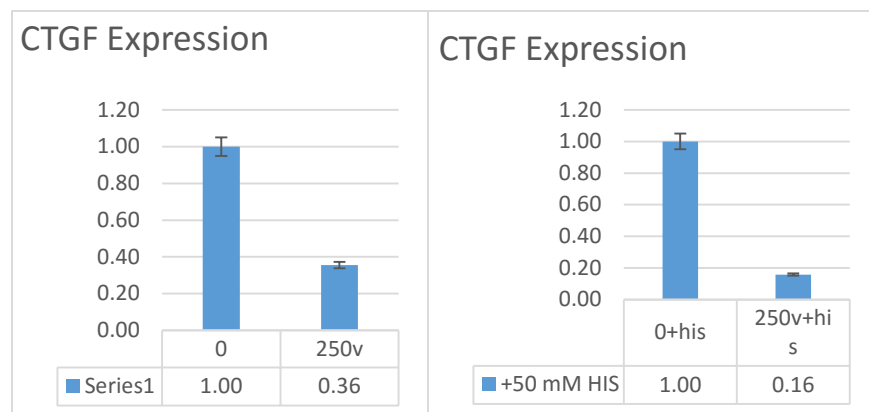


Figure 9. CTGF decreases with the addition of verteporfin, even with singlet oxygens squelched with histidine.

Future Experiments

There are several hypotheses to investigate in order to build upon and validate the results that were generated in the course of my studies. The first hypothesis that should be looked into is testing if antioxidant treatment negates verteporfin's deleterious effects on NSCLC viability. To accomplish this, an MTT and clonogenic assay should be repeated using histidine in conjunction with verteporfin. The results would help distinguish if verteporfin is acting on viability through the hippo pathway or from protein crosslinkage. Secondly, verteporfin may be more effective in limiting stem cell populations than other therapies. To see if this would be a viable pursuit, NSCLC stem cells should be exposed to verteporfin followed by conducting the ALDEFLUOR assay. In doing so, it can be seen if verteporfin affects the population of cells expressing ALDH1A1 (stem-like cells). Also, going back into the folate-conjugate can be looked into in the H460 cells. By competing the folate-verteporfin conjugates intake with folate, deciphering whether the folate-verteporfin conjugate is effective might be plausible. Lastly, verteporfin may be more effective *in vivo* than *in vitro*. To study this, mouse models would need to be developed. It would be costly and time consuming, but the results should yield definitive data on whether verteporfin would be effective or not *in vivo* for NSCLC.

The MTT and the clonogenic assay should be repeated with histidine present, as a singlet oxygen squelcher, during verteporfin exposure. In doing so, it could be determined which modality of verteporfin is responsible for the decrease seen in viability. If the cells are more sensitive to verteporfin treatment without histidine, then protein crosslinking would be suggested to play a bigger role in cell death. On the other hand, if there were no changes in sensitivity, it would be suggested that viability is effected

strictly from the Hippo pathway. While further study would be needed to say convincingly which is the actual culprit, this experiment would be a good indicator at which is responsible in NSCLC.

Another avenue to be looked at is how stem cells are affected with treatment of verteporfin. To accomplish this, the ALDEFLUOR assay would be conducted after verteporfin treatment for two days. Based on the relative percentage of stem cells between the untreated versus the treated cells, it could be deduced how much of an impact verteporfin is having on NSCLC stem cells. If verteporfin is effective at limiting stem cell survival, it could potentially be used in conjunction with other therapies against particularly resistant cancers. Stem cells are typically resistant to traditional chemotherapeutics, so having a drug that could target stem cells could be very beneficial.

After finding out that the relative amount of folate receptors on the H460 cell line was very low, furthering study wasn't conducted. However, it may be possible to use the H460 cell line even though there aren't many receptors. Three separate conditions can be set in cell culture; Having cells exposed to the folate-verteporfin conjugate, the other being exposed to the folate-verteporfin conjugate + folate to compete, and a control plate. These conditions would show if the conjugate is effective at delivering specifically through the folate receptor. The results of this experiment would help show if the folate-verteporfin conjugate should be further looked in to.

Lastly, developing a mouse model to test if verteporfin will be effective *in vivo* for NSCLC is paramount if this drug is to go further into clinical trials. SCID mice would be used in this experiment, being injected with human lung adenocarcinoma cells gathered from patient biopsies. Treatment regimens would begin two weeks after

injection. The mice would be treated in sets of three, the regimen being solvent only, verteporfin, and the folate-verteporfin conjugate since the latter may be more soluble if not more effectively taken up by NSCLC cells. If tumors shrink while having no discernable effect on normal tissues for the verteporfin treatments, verteporfin would hold much more weight as being a viable treatment option for NSCLC.

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