# Reducing Cellular Glutathione Concentrations in A549 Lung Adenocarcinoma Cells Using Chemotherapy and High Dose Acetaminophen

*Willis O'Leary and Karina Hiroshige*
*Valley Catholic School and Oregon Episcopal School*

Table of Contents

**Abstract**

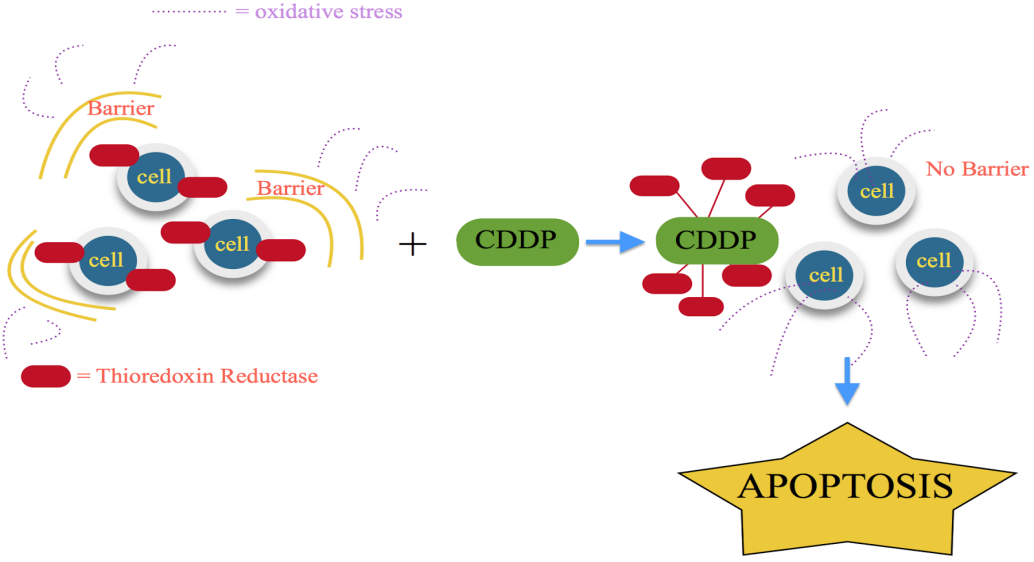
Carcinoma of the lung affects hundreds of thousands of people every year. Lung adenocarcinoma, the most prevalent type of lung cancer, is commonly treated with the chemotherapies cisplatin (CDDP, platinum based agent) and melphalan (MEL, alkylating agent). However, after repeated treatments, the lung adenocarcinoma can become resistant to the chemotherapy. Research shows that resistant lung adenocarcinoma has increased cellular glutathione (GSH) concentrations, suggesting that GSH may be involved in the mechanism of chemoprotection. Therefore, an agent that decreases GSH concentrations may increase chemotherapy cytotoxicity. In the current study, the efficacy of using high dose acetaminophen (AAP, Tylenol) to decrease cellular GSH concentrations and enhance the cytotoxicity of CDDP and MEL was investigated. AAP was found to significantly decrease GSH concentrations when combined with CDDP compared to treatment with CDDP alone. AAP also decreased GSH concentrations when combined with MEL compared to treatment with MEL alone. The cytotoxicity of the AAP and chemotherapy cocktails was also studied, though the data produced were statistically insignificant. The potential synergistic effect of AAP combined with CDDP or MEL on cellular GSH concentrations suggests further investigation may lead to a novel treatment regimen for chemotherapy-resistant lung adenocarcinoma.

## Introduction

Every year, hundreds of thousands of patients are affected with carcinoma of the lung. In 2013 alone, an estimated 159,480 people died of the illness and 228,190 new cases of the disease were reported (National Cancer Institute, 2013). Lung adenocarcinoma is the most prevalent type of lung carcinoma throughout North America (Charloux et al., 1997).

Cisplatin (CDDP, *cis*-diamminedichloroplatinum(II)) is a platinum-based chemotherapeutic agent, widely used to treat lung adenocarcinoma. As a mechanism to trigger cell apoptosis (cell death induced by the cell itself), CDDP blocks a protective protein of the cell called thioredoxin reductase. By eliminating thioredoxin reductase, CDDP damages cell DNA, exposing the cell to oxidative stress (Figure 1). Oxidative stress describes the state in which a cell cannot protect itself against free radicals and toxins.

*Figure 1.* A Mechanism of Cisplatin on Carcinoma Cells. Image rendered by researchers.




However, Zamble and Lippard (1995) demonstrated that cancers such as lung adenocarcinoma can become resistant to CDDP after repeated treatments by various mechanisms such as DNA repair.

Hospers et al. (1998) stated that increased concentrations of the antioxidant glutathione (GSH, γ-L-Glutamyl-L-cysteinylglycine) are found in carcinoma lines that are CDDP-resistant. This phenomenon has also been noted by several other studies (Meijer et al., 1990, Richon et al., 1987, and Waud, 1987). GSH may inhibit CDDP, reducing oxidative stress and increasing cell viability. Therefore, decreasing GSH concentration in lung adenocarcinoma may increase the cytotoxicity of CDDP.

According to Godwin et al. (1992), large doses of acetaminophen (AAP, Tylenol, N-(4-hydroxyphenyl)acetamide) deplete stores of GSH. Neuwelt et al. (2009) found that the addition of AAP to hepatocarcinoma and hepatoblastoma cells can increase the cytotoxicity of CDDP by exhausting the cells' GSH defenses. A combination of CDDP and AAP killed more hepatocarcinoma and hepatoblastoma cells than CDDP alone. In addition, a study by Wu et al. demonstrated that AAP can increase the cytotoxicity of CDDP on human ovarian carcinoma cells. If high doses of AAP can increase the cytotoxicity of CDDP in hepatocarcinoma, hepatoblastoma, and ovarian carcinoma, then AAP may enhance the cytotoxicity of CDDP in lung adenocarcinoma as well.

Melphalan (MEL, 4-[Bis(2-chloroethyl)amino]phenylalanine) is another widely used chemotherapy to treat lung adenocarcinoma. MEL differs from the platinum-based CDDP in that it is an alkylating agent that kills cells by attaching an alkyl group to DNA (Green et al., 1984). To the knowledge of the researchers, the effect of an AAP/MEL cocktail on GSH concentrations and cell viability has never been studied.

AAP may enhance the cytotoxicity of CDDP and MEL to lung adenocarcinoma by decreasing cellular GSH concentrations. Thus, the primary outcome measures of this study are whether CDDP and MEL combined with AAP decreases cellular GSH concentrations and enhances chemotherapy cytotoxicity. Positive results may be the first step to a novel treatment for resistant lung adenocarcinoma.

**Hypothesis**

Treatment with CDDP and/or MEL combined with AAP will result in decreased GSH concentrations and enhanced chemotherapy toxicity to lung adenocarcinoma.

**Materials**

A549 human lung adenocarcinoma cells were used for experimentation and labeled as biohazards. Cells were stored in a sterile culture flask with approximately 20 mL of media in an incubator kept at 37˚C with an atmosphere of 5% $CO_2$. Cells were grown using F12K sterile growth media containing 10% Fetal Bovine Serum, 50 Units/mg of Penicillin-streptomycin antibiotic, and 25 Units/mg of gentamicin antibiotic. A hot-water bath was used to warm media and other solutions before use with cells. The growth media and the CDDP and AAP treatments were stored in a conventional refrigerator. The MEL treatment was stored in a freezer kept at -80˚C (MEL is unstable at higher temperatures). The CytoTox-Glo™ Assay kit (supplied by Promega), QuantiChrom™ Glutathione Assay kit (supplied by BioAssay Systems), and JC-1 dye were stored in a conventional freezer. Approximately 500 mL of the Trypsin (TrypLE) used for cell dissociation was stored in a conventional refrigerator. Cell culture was conducted in a class II biological safety cabinet to prevent contamination. A spray bottle of 50% aqueous ethanol solution was used to sterilize all work surfaces and flasks before use. A 500 mL bottle of 30% bleach aqueous solution was used to kill cells before disposal. All reagents used in the glutathione assay were obtained from a QuantiChrom Glutathione Assay kit.

**Methods: Cell Culture**

Feeding cells:

Before cells were fed, media was warmed in a hot water bath. Every 3-5 days, old media was

transferred to a waste beaker and about 20 mL of new media was added to the original culture flask using sterile technique. The old media was mixed with 30% bleach solution until the solution turned purple, then disposed of down a drain. All pipette tips, paper towels, and centrifuge tubes were discarded in a designated hazardous waste bin.

Splitting cells:

After old growth media was discarded from the original culture flask using sterile technique, about 10 mL of TrypLE was added to coat cells. Cells were incubated for about five minutes or until cells were completely detached from the flask wall (checked with microscope or by eye). Once cells were detached, the solution was transferred into a sterile centrifuge tube and spun for 4 minutes at 4000 rpm. The supernatant was decanted, and cells were resuspended in 20.0 mL of growth media. Cell solution (1.00 mL) was transferred to the original culture flask with about 20 mL of new growth media for a 1:20 split.

**Methods: Glutathione (GSH) Assay (based on a protocol for the QuantiChrom Glutathione Assay Kit by BioAssay Systems)**

1.00 x $10^6$ A549 cells were added with 3.00 mL of media per well in a sterile 6-well culture plate. Cells were incubated at 37˚C with an atmosphere of 5% $CO_2$ for about 2 hours then treated as shown in Table 1. Trials using treatment levels of 5.0 μg/mL produced minimal decrease in GSH concentration, so treatment levels were changed to 10.0 μg/mL.

*Table 1.* Chemotherapy Treatments for GSH Assay

| | |
|---|---|
| Well 1 | Untreated Control (add 10.0 µg/mL media) |
| Well 2 | 10.0 µg/mL CDDP |
| Well 3 | 10.0 µg/mL MEL |
| Well 4 | 10.0 mM AAP |
| Well 5 | 10.0 µg/mL CDDP + 10.0 mM AAP |
| Well 6 | 10.0 µg/mL MEL + 10.0 mM APP |

Using TrypLE, cells were dissociated from each well wall and transferred into sterile individually labeled 15 mL test tubes. Tubes were then centrifuged at 2500 rpm for 5 minutes at 4˚C. The supernatant was carefully decanted, and cells were washed with 7.50 x $10^2$ µL of cold phosphate buffered saline (PBS) then transferred into 1.5 mL centrifuge tubes. These tubes were then centrifuged at 6000 rpm for 2-5 minutes at 4˚C. The supernatant was carefully decanted, and 4.00 x $10^2$ µL of PBS and 1.00 mM ethylenediaminetetraacetic acid (EDTA, pH 6-7) were added to each tube. Tubes were vortexed on high twice then frozen and thawed twice with additional vortexing in between freezes. All tubes were centrifuged at 1400 rpm for 15 minutes at 4˚C. The solutions in these tubes were labeled "samples."

A standard curve concentration was prepared in the following manner. In a sterile 15 mL tube, 4.50 x $10^2$ µL of GSH calibrator and 4.50 x $10^2$ µL of PBS were added. Six 1.5 mL centrifuge tubes were used to conduct a 1:1 serial dilution using the solution created in the 15 mL tube. Two hundred µL from each serial dilution tube was plated in a 96-well plate.

Each sample (2.50 x $10^2$ µL) was mixed with 2.25 x $10^2$ µL of Reagent A in separate 1.5 mL centrifuge tubes then vortexed on high. All tubes were centrifuged at 14,000 rpm for 5 minutes at room

temperature. The supernatant (2.00 x $10^2$ µL) from each tube was transferred into the wells of the 96-well plate previously used (transferring precipitate was avoided). Reagent B (1.0 x $10^2$ µL) was added to each well that contained a solution. The 96-well plate was gently tapped to eliminate bubbles, then a clear sticker was added over the plate to avoid spills. The plate was incubated at room temperature for 20-25 minutes, then the GSH (µM) concentration for each sample was read using a plate reader.

A protein assay (the control) was prepared for GSH. Six centrifuge tubes were used to prepare the various concentrations of bovine serum albumin (BSA), PBS, and PBS-EDTA as directed in Table 2. These solutions were labeled "controls."

*Table 2*. GSH Protein Assay Controls.

| | BSA (µL) | PBS (µL) | PBS-EDTA (µL) | Final Concentration of Protein (mg/mL) |
|---|---|---|---|---|
| Control 1 | 0.0 | 32 | 8.0 | 0 |
| Control 2 | 4.0 | 28 | 8.0 | 0.2 |
| Control 3 | 8.0 | 24 | 8.0 | 0.4 |
| Control 4 | 12 | 20 | 8.0 | 0.6 |
| Control 5 | 16 | 16 | 8.0 | 0.8 |
| Control 6 | 20 | 12 | 8.0 | 1.0 |

The controls (1.00 x 10 µL) were plated in order of concentration in a 96-well plate with duplicates to increase data accuracy. Centrifuge tubes (1.5 mL) were used to hold 5.00 µL of each sample (made previously) with 2.00 µL of PBS. These new samples (1.00 x 10 µL) were then plated in the same 96-well plate with duplicates to increase data accuracy. In a 15 mL tube, 2.00 x $10^{-1}$ mL of Reagent B was mixed with 1.00 x 10 mL of Reagent A. The reagent solution (2.00 x $10^2$ µL) was

added to each well and incubated at 37˚C for 25 minutes, then the GSH (µM) concentration was read for each sample using a plate reader. The GSH assay was repeated four times following the aforementioned protocol.

**Methods: Mitochondrial Membrane Potential (MMP) Assay (using a protocol by Baumber, Ball, Gravance, Medina, and Davies-Morel, 2000)**

Cytotoxicity was determined by measuring the level of mitochondrial membrane potential in the cells. In a 6-well plate, 5.00 x $10^5$ cells were plated in 2.00 mL media per well and incubated at 37˚C with an atmosphere of 5% $CO_2$ for about 2 hours. After allotted time cells were treated as directed in Table 3 using the treatment concentrations specified in Table 4. Treatment levels were determined to be 1.00 x 10 µg/mL and 1.00 x 10 mM after little cell death occurred at lower doses in previous trials.

*Table 3.* Treatments for MMP Assay.

| | |
|---|---|
| Well 1 | Untreated Control (add 1.00 x10 µg/mL media) |
| Well 2 | 10.0 µg/mL CDDP |
| Well 3 | 10.0 µg/mL MEL |
| Well 4 | 10.0 mM AAP |
| Well 5 | 10.0 mM AAP + 10.0 µg/mL CDDP |
| Well 6 | 10.0 mM AAP + 10.0 µg/mL MEL |

*Table 4.* Treatment Concentrations for MMP Assay.

| Treatment Name | Treatment Amount to be Added to Each Well | Final Concentration per Well |
|---|---|---|
| AAP | 5.00 µL of 4M AAP stock | 10.0 mM |
| CDDP | 20.0 µL of 1 mg/mL stock | 10.0 µg/mL |
| MEL | 20.0 µL of 1 mg/mL stock | 10.0 µg/mL |

After treatment, the cells were incubated overnight at 37˚C with an atmosphere of 5% $CO_2$. The next day, JC-1 dye (3.00 µg/mL) and 6.00 x 10 µL of a 30% stock solution of $H_2O_2$ were added to each well (almost) simultaneously. Cells were incubated for 30 minutes at 37˚C with an atmosphere of 5% $CO_2$ then rinsed with 1.00 mL PBS, minimizing extra fluorescence which could have affected the microscopy photos. Cells were held in 1.00 mL PBS for microscopy.

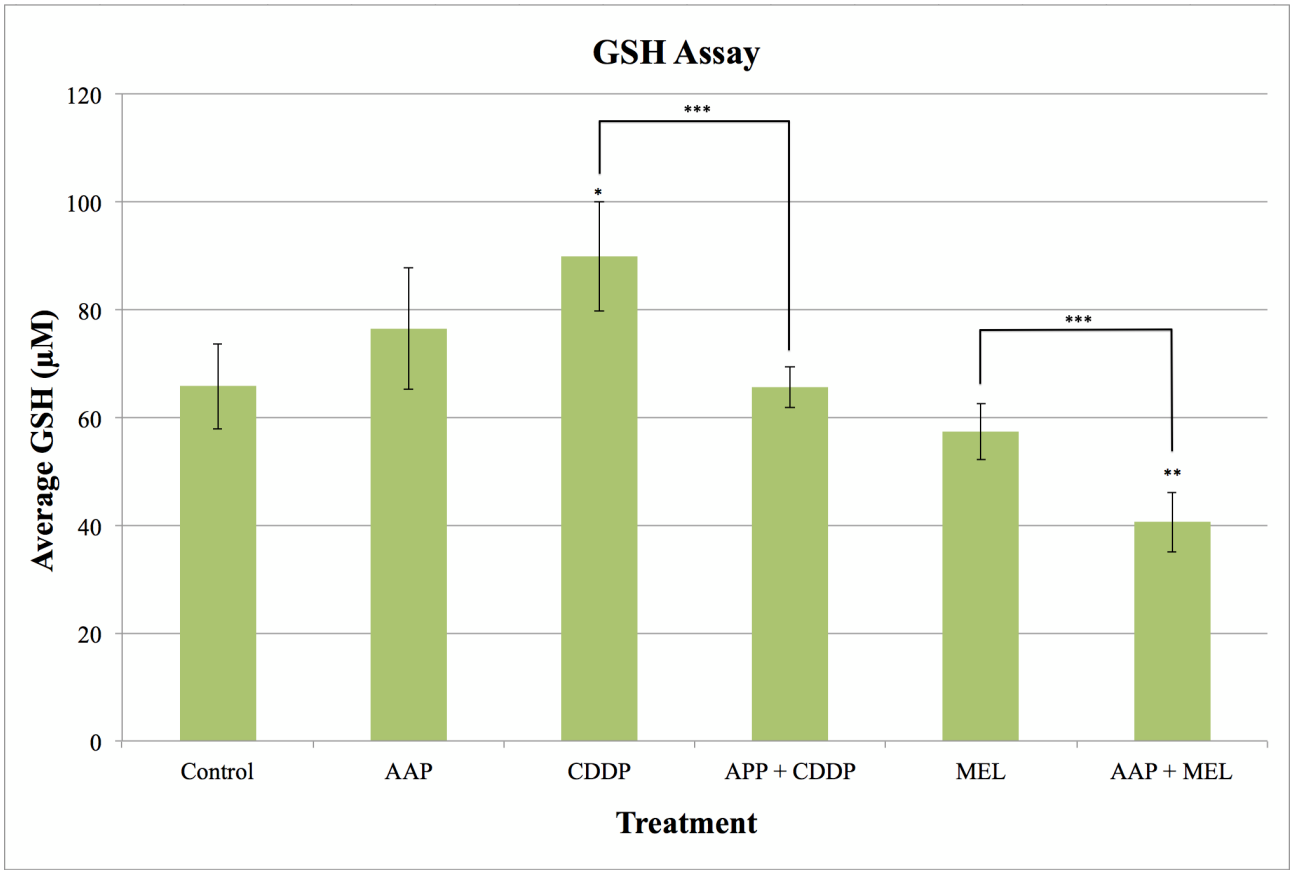
Using a microscope capable of image capture, 10 photos were taken at 200X magnification and 6 photos were taken at 100X magnification of each well. All photos were analyzed for cell viability (green staining indicating dead cells, orange staining indicating live cells) manually using a counting program developed by the researchers. The MMP assay was repeated once following the aforementioned protocol.

The source code for the cell counting program dubbed "CellCount" is available in Appendix C. CellCount was written in the Java programming language using the Eclipse integrated developer environment. CellCount was developed using Java's Swing library and consisted of two classes: MainWindow and ImagePanel. The entry method of the program instantiated a MainWindow object, which handled resources and interacted with the user through a JMenu. Through the JMenu, the user could display an image stored on the file system and edit the current count. MainWindow also held an ImagePanel object. ImagePanel extended JPanel and held the selected JPEG image in a BufferedImage

object. ImagePanel also detected mouse events (by implementing MouseListener) and kept track of the cell count. When a mouse click was detected, ImagePanel marked the click location with either a red square (alive cell, left mouse button) or a green square (dead cell, right mouse button). Alive and dead cells were dynamically tallied and displayed in the window's title bar.

**Results and Analysis**

*Figure 2.* Averaged Glutathione (GSH) Concentrations Graphed with Standard Deviation. Raw data is available in Appendix A.

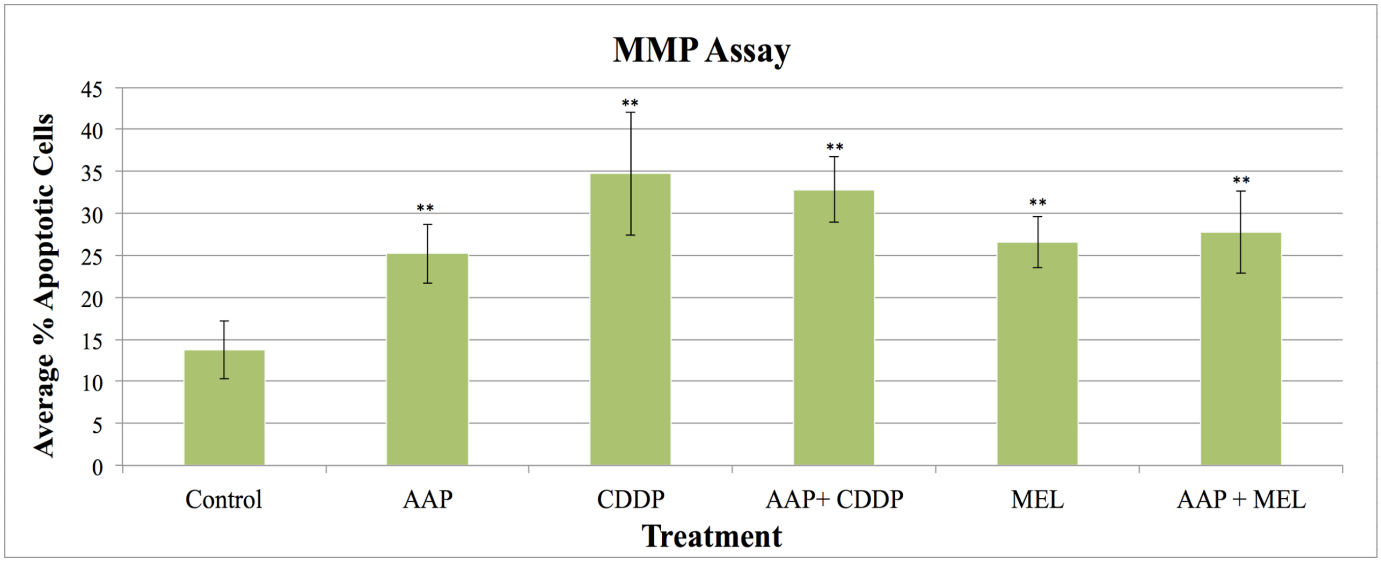



* indicates statistical significance compared to the control ($p<0.05$)
** indicates statistical significance compared to the control ($p<0.01$)
*** indicates statistical significance compared to treatment without AAP ($p<0.01$)

For each chemotherapy treatment eight trials were conducted and cellular GSH concentrations

were evaluated and displayed in Figure 2. The untreated A549 lung adenocarcinoma control had a GSH concentration of 65.8 µM ± 7.9. Treatment with AAP resulted in a slightly higher GSH concentration (76.5 µM ± 11.3) compared to the control. Cells treated with CDDP alone resulted in a higher GSH concentration (89.9 µM ± 10.2) compared to the control. A decrease in GSH concentration is noted when AAP was combined with CDDP (65.6 µM ± 3.80) compared to treatment with CDDP alone (24.3 µM difference). Cells treated with MEL had a slightly lower GSH concentration (57.3 µM ± 5.20) compared to the control. Combining MEL with AAP resulted in the lowest GSH concentration (40.6 µM ± 5.53) compared to all other treatments.

*Figure 3.* Percent Apoptotic Cells Averaged and Graphed from the Mitochondrial Membrane Potential (MMP) Assay with Standard Deviation. Raw data is available in Appendix B.

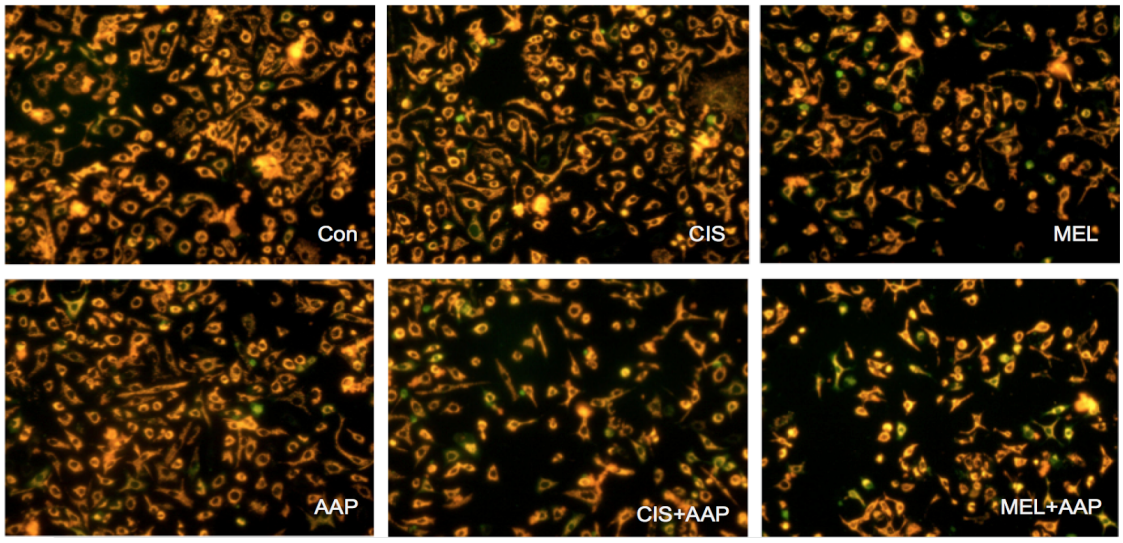



** indicates statistical significance compared to the control ($p<0.01$)

Six MMP photos (at 100X magnification) per treatment were analyzed by eye for cell viability/apoptosis before percent apoptotic cells was calculated, averaged, and shown in Figure 3. A selection of the photos analyzed are available in Figure 4, where orange staining indicates cell viability

and green staining indicates cell apoptosis.

*Figure 4.* Selected Photos of A549 Lung Adenocarcinoma After Being Stained for MMP at 100X Magnification (Con=Control, CIS=CDDP).




Cell apoptosis (14% ± 3.5%) occurred after lung adenocarcinoma cells were left untreated (control). Treatment with AAP resulted in a notable increase in cell death (25% ± 3.5%) compared to the control and is displayed in Figure 4 as green cells (indicating cell apoptosis). Treatment with CDDP resulted in a significant increase in cell death (35% ± 6.8%) compared to the control and is shown with several green cells in Figure 4. Treatment with MEL resulted in a small increase in cell death (27% ± 3.0%) compared to the control and is shown with a few green cells in Figure 4. The addition of AAP to both CDDP and MEL treatments resulted in a significant increase in cell death (33% ± 3.9% and 28% ± 4.9%, respectively) compared to the control.

**Discussion of Results**

The efficacy of using AAP to enhance the cytotoxicity of CDDP and MEL on A549 lung adenocarcinoma was studied by evaluating cellular GSH concentrations and analyzing MMP to

determine cell viability. The results validated the hypothesis that AAP combined with either CDDP or MEL would reduce cellular GSH concentrations. However, experiments investigating chemotherapy enhancement by the addition of AAP (via percent cell apoptosis) produced inconclusive results.

Cellular GSH concentrations were evaluated and displayed in Figure 2. Treatment with AAP alone resulted no significant change ($p>0.05$) in GSH concentration compared to the control. Treatment with CDDP produced a significant ($p<0.01$) increase (24.0 μM) in GSH concentration. This increase was unexpected and the mechanism remains unknown; however, the increase may have been due to the cells producing excess antioxidants (like GSH) in an attempt to protect themselves against oxidative stress.

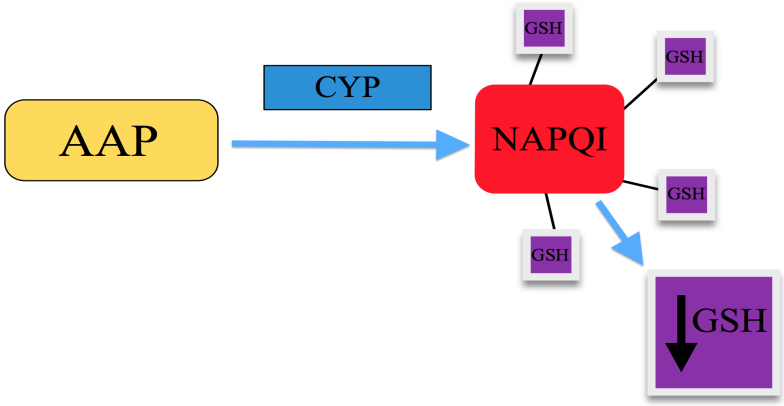
The addition of AAP to CDDP provided a significant ($p<0.01$) decrease (24.3 μM) in GSH concentration compared to CDDP treatment alone. However, this large decrease in GSH concentration as a result of the addition of AAP was statistically insignificant ($p>0.1$) compared to the control. This result differs from the findings of Neuwelt et al. (2009) where a combination treatment of AAP and CDDP significantly decreased GSH concentrations compared to the control in hepatocarcinoma and hepatoblastoma cells. Therefore, the results of the current study suggest that although the addition of AAP to CDDP notably decreases GSH concentrations, this combination treatment may not be as effective in treating lung adenocarcinoma as hepatocarcinoma and hepatoblastoma. Additionally, research by Rudin et al. (2003) suggests that increased GSH concentrations are involved in the mechanism of CDDP-resistance in B-cell lymphoma 2 carcinoma. Thus, since GSH concentrations were greatly decreased with a combination treatment of AAP and CDDP in the current study, this treatment regimen may be beneficial to alleviate CDDP-resistance in B-cell lymphoma 2. The results of the current study warrant further investigation into the efficacy of using a chemotherapy cocktail of AAP

and CDDP to reduce GSH concentration in alternative carcinoma lines.

Treatment with MEL alone resulted in no significant ($p>0.05$) change in GSH concentration. The chemotherapy cocktail of MEL and AAP produced a significant ($p<0.01$) decrease (25.2 μM) in GSH concentration compared to the control and treatment with MEL alone (16.7 μM, $p<0.01$). By drastically decreasing GSH concentration, the combination treatment of AAP and MEL likely would have increased the cell's sensitivity to toxic agents and enhanced chemotherapy cytotoxicity. Therefore, a cocktail of AAP and MEL may be an effective treatment to enhance chemotherapy cytotoxicity in resistant lung adenocarcinoma.

While treatment with AAP alone resulted in no significant change in GSH concentrations, AAP combined with CDDP or MEL significantly decreased GSH concentrations, suggesting there is a possible synergistic effect between AAP and CDDP or MEL. A potential explanation centers on the cancer cell's cytochrome P450 system. As shown in Figure 5, cytochrome P450 breaks down AAP into the toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Hinson, Roberts, & James, 2010). Cytochrome P450 is present in reduced levels in lung cells as compared to liver cells (Somers et al., 2007). This suggests AAP has reduced metabolism in lung cells. Since CDDP is known to increase levels of cytochrome P450 (Florea and Büsselberg, 2011), we postulate CDDP increases the cytochrome P450 catalyzed breakdown of AAP to NAPQI in the lung adenocarcinoma cells. NAPQI then binds with GSH, thereby reducing the GSH concentrations (Figure 5).

*Figure 5.* Mechanism of AAP on GSH Concentrations. (CYP= Cytochrome P450 system)




MMP was measured to determine cell viability after various treatments. The hypothesis was that cell viability would decrease when all treatments were applied compared to the control, and that the addition of AAP to CDDP and/or MEL would result in the highest percent cell apoptosis. Results showed that compared to the control, all treatments significantly increased cell death as shown in Figure 3 and suggested by photos in Figure 4. When comparing the effectiveness of the MEL versus CDDP, the results showed that CDDP induced a significantly higher percent cell death (difference of 7.3%, $p<0.05$). No conclusions can be made as to whether MEL cytotoxicity was enhanced by AAP because the results were statistically insignificant ($p>0.1$). Also unclear is the effectiveness of CDDP compared to the combination treatment of CDDP and AAP because data were statistically insignificant ($p>0.1$). In summary, statistical insignificance and high standard deviation prevent the MMP data from revealing any noteworthy trends, aside from the fact that all the treatments provided some increase in cell death compared to no treatment. This inconclusive data can be attributed to small sample sizes in individual pictures as well as the variability of counting hundreds of cells by eye. To aid in manual cell counting, a Java program was developed. The program made the counting process much faster and alleviated the difficulty of remembering which cells had been counted. However, human error was still inevitable because some cells were stained partially orange (live) and green (dead), indicating that the cell was still

undergoing apoptosis when the photo was taken. Thus, the researchers had to try to objectively decide whether to classify those cells as dead or alive. More trials would have been conducted to increase data accuracy, however due to time constraints only one MMP assay was completed (even with computer aid cell counting took over 10 hours).

Using high dose AAP was required to decrease GSH concentration appreciably in lung adenocarcinoma. While the significant decrease in GSH concentration displayed when AAP is combined with MEL is a favorable outcome, the large dosage of AAP required to induce a decrease in GSH concentration would produce severe side effects, such as liver failure, in patients. Therefore, a rescue drug to halt the toxic effects of AAP on the human body is required if chemotherapy enhancement with high dose AAP is to become a viable treatment. N-acetylcysteine (NAC) is commonly used to treat AAP overdose. NAC is an antioxidant, meaning that it fights oxidative stress. NAC inhibits the adversarial effects of AAP, stopping cell apoptosis. Additionally, NAC has been shown to halt CDDP induced apoptosis through the caspase signalling pathway (Wu, Muldoon, & Neuwelt, 2005). Thus, NAC is beneficial in not only blocking the dangerous side effects of AAP, but also CDDP. Further experiments should test whether NAC offers chemoprotection against CDDP and/or MEL combined with AAP in lung adenocarcinoma.

Lung adenocarcinoma is the most prevalent type of lung carcinoma, affecting hundreds of thousands of patients every year. Treatments such as CDDP and MEL are widely used to treat lung adenocarcinoma; however, if the cancer becomes resistant to chemotherapy treatment there are few options to help enhance cytotoxicity. In this study, AAP combined with CDDP or MEL significantly reduced GSH concentrations, suggesting that cells became more susceptible to the chemotherapeutic agents. Thus, an increase in cell death when AAP was used in combination with CDDP and MEL was

hypothesised. However, due to statistical error, further investigation is required to draw conclusions as to whether AAP enhances the cytotoxicities of CDDP and MEL. In future study if AAP is found to decrease cell viability when combined with CDDP or MEL, then a novel treatment for resistant lung adenocarcinoma using AAP may be viable.

**Conclusion**

High dose AAP combined with CDDP or MEL significantly reduced cellular GSH concentrations in A549 lung adenocarcinoma. No conclusion could be drawn regarding enhanced chemotherapy cytotoxicity.

**Acknowledgements**

We would like to formally thank our mentor, Dr. Leslie L. Muldoon, for guiding us throughout the research process, from writing our first journal club to finishing our own report. We deeply appreciate Kathleen Beeson for teaching us laboratory skills and techniques. Dr. Jeffrey Wu, for showing us how to perform assays and proper pipetting technique. Emily Miller and Esther Kim for teaching us how to do a GSH assay. Additional thanks to Heather Mcconnell, Josh Robertson, and Sangeet Lal.

We would also like to thank our parents for their "research grants" that continue to support our survival, as well as their wisdom. Finally, we would like to extend our gratitude to Bevin Daglen for being our adult sponsor and giving us advice and feedback on our report.

**References**

Ashtiani, H. R. A., Bakhshandi, A. K., Rahbar, M., Mirzaei, A., Malekpour, A., and Rastegar, H. (2011). Glutathione, cell proliferation and differentiation. *African Journal of Biotechnology, 10*(34), 6348-6363.

Baumber, J., Ball, B. A., Gravance, C. G., Medina, V., and Davies-More, M. C. G. (2000). The effect of reactive oxygen species on equine sprem motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology, 21*(6), 895-902.

Centers of Disease Control and Prevention. (2011). *Rates of new lung cancer cases*. Retrieved from http://www.cdc.gov/Features/dsLungCancer/.

Charloux, A., Quoix, E., Wolkove, N., Small, D., Pauli, G., Kreisman, H. (1997). The increasing incidence of lung adenocarcinoma: reality or artefact? A review of the epidemiology of lung adenocarcinoma. *International Journal of Epidemiology, 26*(1), 14-23.

Florea, A. & Büsselberg, D. (2011). Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. *Cancers, 3*, 1351-1371.

Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C., and Anderson, M. E. (1992). High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proceedings of the National Academy of Sciences, 89*, 3070–3074.

Green, J. A., Vistica, D. T., Young, R. C., Hamilton, T. C., Rogan, and A. M., Ozols, R. F. (1984). Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. *Cancer Research, 44*, 5427-5431.

Hinson, J. A., Roberts, D. W., and James, L. P. (2010). Mechanisms of acetaminophen-induced liver necrosis. *Handbook of Experimental Pharmacology, 196*, 369-405).

Hospers, G. A., Mulder, N. H., de Jong, B., de Ley, L., Uges, D. R., Fichtinger-Schepman, A. M., Scheper, R. J., and de Vries, E. G. (1988). Characterization of a human small cell lung carcinoma cell line with acquired resistance to cis-diamminedichloroplatinum (II) in vitro. *Cancer Res, 48*(23), 6803–6807.

Mayo Clinic. (n.d.) *Lung cancer*. Retrieved from http://www.mayoclinic.org/lung-cancer/types.html.

Meijer, C., Mulder, N. H., Hospers, G. A. P., Uges, D. R. A., and de Vries, E. G. E. (1990). The role of glutathione in resistance to cisplatin in a human small cell lung cancer cell line. *British Journal of Cancer, 62,* 72-77.

Nakagawa, K., Yokota, J., Wada, M., Sasaki, Y., Fujiwara, Y., Sakai, M., Muramatsu, M., Terasaki, T., Tsunokawa, Y., Terada, M., et al. (1988). Levels of glutathione S transferase pi mRNA in

human lung cancer cell lines correlate with the resistance to cisplatin and carboplatin. *Jpn J Cancer Res, 79*(3), 301–304.

National Cancer Institute. (2013). Lung cancer. In *National Cancer Institute*. Retrieved from *http://www.cancer.gov/cancertopics/pdq/treatment/non-small-cell-lung/healthprofessional/page1*.

Neuwelt, A. J., Wu, J. Y., Knap, N., Losin, M., Neuwelt, E. A., Pagel, M. A., Warmann, S., Fuchs, J., Czauderna, P., and Wozniak M. (2009). Using acetaminophen's toxicity mechanism to enhance cisplatin efficacy in hepatocarcinoma and hepatoblastoma cell lines. *Neoplasia, 11*(10), 1003-1011.

Richon, V. M., Schulte, N., and Eastman, A. (1987). Multiple mechanisms of resistance to af-Diamminedichloroplatinum(II) in murine leukemia LI 210 cells. *Cancer Research, 47*, 2056-2061.

Rudin, C. M., Yang, Z., Schumaker, L. M., VanderWeele, D. J., Newkirk, K., Egorin, M. J., Zuhowski, E. G., and Cullen, K. J. (2003). Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance. *Cancer Research, 63*, 312-318.

Somers, G. I., Lindsay, N., Lowdon, B. M., Jones, A. E., Freathy, C., Ho, S., et al. (2007). A comparison of the expression and metabolizing activities of phase I and II enzymes in freshly isolated human lung parenchymal cells and cryopreserved human hepatocytes. *Drug Metabolism and Disposition, 35*(10), 1797-1805.

Waud, W. R. (1987). Differential uptake of c/s-Diamminedichloroplatinum(II) by sensitive and resistant murine LI 210 leukemia cells. *Cancer Research, 47*, 6549-6555.

Web MD. (2010). *Lung Cancer- Medications*. Retrieved from http://www.mayoclinic.org/lung-cancer/types.html.

Wu, Y. J., Neuwelt, A. J., Muldoon, L. L., and Neuwelt, E. A. (2012) Acetaminophen enhances the chemotherapeutic effect of cisplatin and paclitaxel on human ovarian carcinoma cells in vitro. *Cancer Research, 72*(8), 8 Suppl.

Wu, Y. J., Muldoon, L. L., Neuwelt, E. A. (2005). The chemoprotective agent *N-Acetylcysteine* blocks cisplatin-induced apoptosis through caspase signaling pathway. *The Journal of Pharmacology and Experimental Therapeutics, 312*(2), 424-431.

Zamble, D. B., and Lippard, S. J., (1995). Cisplatin and DNA repair in cancer chemotherapy. *Trends in Biochemical Sciences, 10*(10), 435-439.

## Appendix A: GSH Raw Data

| Treatment - Trial | Protein Value | GSH Value | GSH Conc. ( µM) | Mean GSH Conc. (µM) | Standard Deviation (µM) | P(Control) | P(CDDP) | P(MEL) |
|---|---|---|---|---|---|---|---|---|
| **Control - 1** | 0.795 | 51.322 | 64.55597 | 65.79901 | 7.92380 | n/a | n/a | n/a |
| **Control - 2** | 0.815 | 54.476 | 66.84172 | | | | | |
| **Control - 3** | 0.78 | 43.96 | 56.35897 | | | | | |
| **Control - 4** | 0.805 | 45.012 | 55.91553 | | | | | |
| **Control - 5** | 0.785 | 57.656 | 73.44713 | | | | | |
| **Control - 6** | 0.91 | 55.07 | 60.51648 | | | | | |
| **Control - 7** | 0.83 | 64.116 | 77.24819 | | | | | |
| **Control - 8** | 0.74 | 52.916 | 71.50811 | | | | | |
| **AAP - 1** | 0.86 | 53.776 | 62.53023 | 76.49837 | 11.26224 | 0.01073 | n/a | n/a |
| **AAP - 2** | 0.845 | 55.528 | 65.71361 | | | | | |
| **AAP - 3** | 0.825 | 59.034 | 71.55636 | | | | | |
| **AAP - 4** | 0.825 | 59.734 | 72.40485 | | | | | |
| **AAP - 5** | 0.8 | 64.116 | 80.14500 | | | | | |
| **AAP - 6** | 0.77 | 60.67 | 78.79221 | | | | | |
| **AAP - 7** | 0.73 | 71.87 | 98.45205 | | | | | |
| **AAP - 8** | 0.82 | 67.562 | 82.39268 | | | | | |
| **CDDP - 1** | 0.85 | 69.198 | 81.40941 | 89.87604 | 10.22426 | 0.00001 | n/a | n/a |
| **CDDP - 2** | 0.835 | 68.848 | 82.45269 | | | | | |
| **CDDP - 3** | 0.85 | 70.25 | 82.64706 | | | | | |
| **CDDP - 4** | 0.815 | 66.394 | 81.46503 | | | | | |
| **CDDP - 5** | 0.76 | 76.178 | 100.2421 | | | | | |
| **CDDP - 6** | 0.855 | 77.47 | 90.60819 | | | | | |
| **CDDP - 7** | 0.82 | 89.532 | 109.8537 | | | | | |
| **CDDP - 8** | 0.785 | 71.44 | 91.00637 | | | | | |
| **AAP + CDDP - 1** | 0.83 | 56.58 | 68.16867 | 65.58668 | 3.80287 | 0.95010 | 0.00085 | n/a |
| **AAP + CDDP - 2** | 0.83 | 57.28 | 69.01205 | | | | | |
| **AAP + CDDP - 3** | 0.84 | 56.93 | 67.77381 | | | | | |
| **AAP + CDDP - 4** | 0.775 | 52.724 | 68.03097 | | | | | |
| **AAP + CDDP - 5** | 0.775 | 52.056 | 67.16903 | | | | | |
| **AAP + CDDP - 6** | 0.81 | 46.886 | 57.88395 | | | | | |
| **AAP + CDDP - 7** | 0.8 | 50.762 | 63.45250 | | | | | |
| **AAP + CDDP - 8** | 0.81 | 51.194 | 63.20247 | | | | | |
| **MEL - 1** | 0.795 | 40.106 | 50.44780 | 57.30857 | 5.19706 | 0.01417 | n/a | n/a |
| **MEL - 2** | 0.78 | 40.456 | 51.86667 | | | | | |
| **MEL - 3** | 0.785 | 42.91 | 54.66242 | | | | | |
| **MEL - 4** | 0.785 | 42.91 | 54.66242 | | | | | |
| **MEL - 5** | 0.8 | 49.47 | 61.83750 | | | | | |
| **MEL - 6** | 0.775 | 47.748 | 61.61032 | | | | | |
| **MEL - 7** | 0.78 | 45.594 | 58.45385 | | | | | |
| **MEL - 8** | 0.815 | 52.916 | 64.92761 | | | | | |
| **AAP + MEL - 1** | 0.78 | 26.786 | 34.34103 | 40.58567 | 5.52720 | 0.00017 | n/a | 0.00002 |
| **AAP + MEL - 2** | 0.78 | 24.332 | 31.19487 | | | | | |
| **AAP + MEL - 3** | 0.795 | 32.394 | 40.74717 | | | | | |
| **AAP + MEL - 4** | 0.805 | 35.548 | 44.15901 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **AAP + MEL - 5** | 0.78 | 35.686 | 45.75128 | | | | | |
| **AAP + MEL - 6** | 0.775 | 36.116 | 46.60129 | | | | | |
| **AAP + MEL - 7** | 0.795 | 34.394 | 43.26289 | | | | | |
| **AAP + MEL - 8** | 0.79 | 30.516 | 38.62785 | | | | | |

**Key**
Conc. = Concentration
P(Control) = P value compared to control
P(CDDP) = P value compared to CDDP treatment
P(MEL) = P value compared to MEL treatment

## Appendix B: MMP Raw Data

| Treatment - Photo | Alive Cells | Dead Cells | Percent Dead | Mean Percent Dead | Standard Deviation | P(Control) | P(CDDP) | P(MEL) |
|---|---|---|---|---|---|---|---|---|
| **Control - 1** | 87 | 19 | 17.9245 | 13.7603 | 3.4693 | n/a | n/a | n/a |
| **Control - 2** | 101 | 20 | 16.5289 | | | | | |
| **Control - 3** | 91 | 14 | 13.3333 | | | | | |
| **Control - 4** | 129 | 23 | 15.1316 | | | | | |
| **Control - 5** | 114 | 11 | 8.8000 | | | | | |
| **Control - 6** | 74 | 9 | 10.8434 | | | | | |
| **AAP - 1** | 77 | 34 | 30.6306 | 25.2003 | 3.5002 | 0.0017 | n/a | n/a |
| **AAP - 2** | 124 | 34 | 21.5190 | | | | | |
| **AAP - 3** | 152 | 41 | 21.2435 | | | | | |
| **AAP - 4** | 128 | 44 | 25.5814 | | | | | |
| **AAP - 5** | 111 | 38 | 25.5034 | | | | | |
| **AAP - 6** | 85 | 31 | 26.7241 | | | | | |
| **CDDP - 1** | 114 | 46 | 28.7500 | 34.7384 | 6.8494 | 0.0009 | n/a | n/a |
| **CDDP - 2** | 71 | 54 | 43.2000 | | | | | |
| **CDDP - 3** | 70 | 47 | 40.1709 | | | | | |
| **CDDP - 4** | 93 | 48 | 34.0426 | | | | | |
| **CDDP - 5** | 78 | 46 | 37.0968 | | | | | |
| **CDDP - 6** | 110 | 37 | 25.1701 | | | | | |
| **AAP + CDDP - 1** | 81 | 50 | 38.1679 | 32.7982 | 3.8935 | $2.25*10^{-6}$ | 0.5506 | n/a |
| **AAP + CDDP - 2** | 89 | 53 | 37.3239 | | | | | |
| **AAP + CDDP - 3** | 104 | 46 | 30.6667 | | | | | |
| **AAP + CDDP - 4** | 68 | 31 | 31.3131 | | | | | |
| **AAP + CDDP - 5** | 81 | 34 | 29.5652 | | | | | |
| **AAP + CDDP - 6** | 85 | 36 | 29.7521 | | | | | |
| **MEL - 1** | 113 | 37 | 24.6667 | 26.5418 | 3.0436 | 0.0014 | n/a | n/a |
| **MEL - 2** | 72 | 28 | 28.0000 | | | | | |
| **MEL - 3** | 100 | 34 | 25.3731 | | | | | |
| **MEL - 4** | 70 | 28 | 28.5714 | | | | | |
| **MEL - 5** | 82 | 36 | 30.5085 | | | | | |
| **MEL - 6** | 95 | 27 | 22.1311 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **AAP + MEL - 1** | 84 | 35 | 29.4118 | 27.8029 | 4.9107 | 0.0022 | n/a | 0.4378 |
| **AAP + MEL - 2** | 44 | 21 | 32.3077 | | | | | |
| **AAP + MEL - 3** | 110 | 30 | 21.4286 | | | | | |
| **AAP + MEL - 4** | 68 | 24 | 26.0870 | | | | | |
| **AAP + MEL - 5** | 78 | 40 | 33.8983 | | | | | |
| **AAP + MEL - 6** | 87 | 27 | 23.6842 | | | | | |

**Key**
Conc. = Concentration
P(Control) = P value compared to control
P(CDDP) = P value compared to CDDP treatment
P(MEL) = P value compared to MEL treatment

## Appendix C: CellCount Source Code

```
//--------------------------------------------------------------------
// File:        MainWindow.java
// Purpose:    Runs the main window using the swing gui. Handles
//     resource loading as well as menus.
// Author:      Will O'Leary
// Version:     0.2
// Files:      Let [Root] be the filepath of the executable,
//              [Root]/data/alive.jpg and [Root]/data/dead.jpg
//              must exist.
//
// This file is part of CellCount.
//
// CellCount is free software: you can redistribute it and/or modify
// it under the terms of the GNU General Public License as published by
// the Free Software Foundation, either version 3 of the License, or
// (at your option) any later version.
//
// CellCount is distributed in the hope that it will be useful,
// but WITHOUT ANY WARRANTY; without even the implied warranty of
// MERCHANTABILITY or FITNESS FOR A PARTICULAR PURPOSE.  See the
// GNU General Public License for more details.
//
// You should have received a copy of the GNU General Public License
// along with CellCount.  If not, see <http://www.gnu.org/licenses/>.
//--------------------------------------------------------------------

import javax.swing.*;
import java.awt.event.*;
import java.awt.*;
import java.awt.image.*;
import java.io.*;
import javax.imageio.*;
import java.net.*;

import static javax.swing.JOptionPane.*;

public class MainWindow implements ActionListener{

   private static JFrame frame;
   private JMenuItem menuItem[] = new JMenuItem[5];
   private JFileChooser dialog = new JFileChooser();
   private ImagePanel panel = new ImagePanel();
```

```
private JLabel imageLabel = new JLabel();

private BufferedImage image;
private Dimension dim;

public MainWindow() {
   if (frame == null)
   {
       dim = Toolkit.getDefaultToolkit().getScreenSize();
       frame = new JFrame();
       frame.setSize((int) dim.getWidth(),
              (int) dim.getHeight()-30);
       JMenuBar menuBar = new JMenuBar();
       JMenu menu = new JMenu("Menu");
       frame.add(menuBar,BorderLayout.NORTH);
       menuBar.add(menu);
       menuItem[0] = new JMenuItem("Load Image");
       menuItem[1] = new JMenuItem("Minus Alive");
       menuItem[2] = new JMenuItem("Plus Alive");
       menuItem[3] = new JMenuItem("Minus Dead");
       menuItem[4] = new JMenuItem("Plus Dead");
       menuItem[0].addActionListener(this);
       menuItem[1].addActionListener(this);
       menuItem[2].addActionListener(this);
       menuItem[3].addActionListener(this);
       menuItem[4].addActionListener(this);
       menu.add(menuItem[0]);
       menu.add(menuItem[1]);
       menu.add(menuItem[2]);
       menu.add(menuItem[3]);
       menu.add(menuItem[4]);
       frame.setJMenuBar(menuBar);
       frame.setDefaultCloseOperation(JFrame.DISPOSE_ON_CLOSE);
       frame.setTitle("Cell Count -- No Image Loaded");
       frame.setResizable(false);
       frame.setLayout(null);
       panel.setLayout(null);
       panel.setLocation(0,0);
       panel.setSize(dim);
       imageLabel.setLocation(0,0);
       frame.add(panel);
       panel.add(imageLabel);
       panel.setComponentZOrder(imageLabel,0);
       frame.setVisible(true);
   }
   frame.repaint();
}

public void readInFile(String fileName) {
   File file = new File(fileName);
   if(file.isFile()) {
       try {
     image = ImageIO.read(file);
       } catch (IOException e) {
     showMessageDialog(frame,"No file found!",
                       "No file read or found",INFORMATION_MESSAGE);
     e.printStackTrace();
       }
   }
   else {
       URL url = getClass().getResource(fileName);
       if (url == null) {
     try {
         url = new URL(fileName);
     } catch (MalformedURLException e) {
         showMessageDialog(frame,"No file found!",
```

```
                        "No Image file or found",INFORMATION_MESSAGE);
          e.printStackTrace();
      }
        }
        try {
      image = ImageIO.read(url);
        } catch (IOException e) {
      showMessageDialog(frame,"No file found!","No Image file",WARNING_MESSAGE);
      e.printStackTrace();
        }
    }
}

public void setImage(){
   panel.removeAll();
   double scaledWidth;
   double scaledHeight;
   double w = image.getWidth();
   double h = image.getHeight();
   double wScreen = dim.getWidth()-20;
   double hScreen = dim.getHeight()-100;
   double imageRatio = w/h;
   double screenRatio = wScreen/hScreen;
   if(imageRatio<=screenRatio){
       scaledHeight = hScreen;
       scaledWidth = scaledHeight * imageRatio;
   }
   else{
       scaledWidth = wScreen;
       scaledHeight = scaledWidth/imageRatio;
   }
   ImageIcon icon = new
   ImageIcon(image.getScaledInstance((int)scaledWidth,(int)scaledHeight,Image.SCALE_DEF
   AULT));
   imageLabel.setSize((int) scaledWidth, (int) scaledHeight);
   imageLabel.setIcon(icon);
   ImagePanel.aliveCount = 0;
   ImagePanel.deadCount = 0;
   panel.add(imageLabel);
   panel.updateUI();
}

public void actionPerformed(ActionEvent e) {
   if(e.getSource()==menuItem[0]) {
       if(dialog.showOpenDialog(null)==(JFileChooser.APPROVE_OPTION)) {
     readInFile(dialog.getSelectedFile().getAbsolutePath());
     if(image==null) {
         showMessageDialog(frame,"Does not compute !","No Image
   file",INFORMATION_MESSAGE);
     }
     else {
         setImage();
     }
       }
   }
   else if(e.getSource()==menuItem[1]) {
       ImagePanel.aliveCount--;
   }
   else if(e.getSource()==menuItem[2]) {
       ImagePanel.aliveCount++;
   }
   else if(e.getSource()==menuItem[3]){
       ImagePanel.deadCount--;
   }
   else if(e.getSource()==menuItem[4]){
       ImagePanel.deadCount++;
```

```
      }
      updateTitle();
   }
   public static void main(String[] arg) {
      new MainWindow();
   }
   public static void updateTitle(){
      frame.setTitle("Cell Count -- Alive: "+ImagePanel.aliveCount+" -- Dead:
      "+ImagePanel.deadCount);
   }
}

//-----------------------------------------------------------------
// File:        ImagePanel.java
// Purpose:    Holds the image and handles cell counting and marking
// Author:      Will O'Leary
// Version:     0.2
//
// This file is part of CellCount.
//
// CellCount is free software: you can redistribute it and/or modify
// it under the terms of the GNU General Public License as published by
// the Free Software Foundation, either version 3 of the License, or
// (at your option) any later version.
//
// CellCount is distributed in the hope that it will be useful,
// but WITHOUT ANY WARRANTY; without even the implied warranty of
// MERCHANTABILITY or FITNESS FOR A PARTICULAR PURPOSE.  See the
// GNU General Public License for more details.
//
// You should have received a copy of the GNU General Public License
// along with CellCount.  If not, see <http://www.gnu.org/licenses/>.
//-----------------------------------------------------------------

import java.awt.Toolkit;
import java.awt.event.MouseEvent;
import java.awt.event.MouseListener;
import java.io.File;
import java.io.IOException;
import java.net.URL;

import javax.imageio.ImageIO;
import javax.swing.ImageIcon;
import javax.swing.JLabel;
import javax.swing.JPanel;


public class ImagePanel extends JPanel implements MouseListener {

   private static final long serialVersionUID = 1L;

   static int aliveCount;
   static int deadCount;

   ImageIcon alive;
   ImageIcon dead;

   public ImagePanel(){
      super();
      addMouseListener(this);
      URL urlImage = getClass().getResource("/data/alive.jpg");
      alive = new ImageIcon(urlImage);
      urlImage = getClass().getResource("/data/dead.jpg");
      dead = new ImageIcon(urlImage);
   }
```

```
   public void mousePressed(MouseEvent e) {}
   public void mouseReleased(MouseEvent e) {
      JLabel newLabel;
      int button = e.getButton();
        if(button == MouseEvent.BUTTON1){
            aliveCount++;
            newLabel = new JLabel();
            newLabel.setSize(8,8);
            newLabel.setLocation(e.getX(),e.getY());
            newLabel.setVisible(true);
            add(newLabel);
            setComponentZOrder(newLabel,0);
            newLabel.setIcon(alive);
            updateUI();
            revalidate();
            repaint();
        }
        else if(button == MouseEvent.BUTTON3){
            deadCount++;
            newLabel = new JLabel(dead);
            newLabel.setSize(8,8);
            newLabel.setLocation(e.getX(),e.getY());
            newLabel.setVisible(true);
            add(newLabel);
            setComponentZOrder(newLabel, 0);
            updateUI();
            revalidate();
            repaint();
        }
        MainWindow.updateTitle();
   }
   public void mouseEntered(MouseEvent e) {}
   public void mouseExited(MouseEvent e) {}
   public void mouseClicked(MouseEvent e) {}
}
```