DEFINING THE RADIATION SENSITIZING ACTIVITY OF THE TUBULIN DEPOLYMERIZING AGENT N-ACETYL COLCHICINOL (NAC)

By

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Abstract:

The goal of this study was to examine the potential for the drug N-acetyl colchicinol (NAC) to be used to increase the efficacy of radiation therapy in the treatment of rectal cancer. In initial studies we defined the maximum non-cytotoxic dose of NAC and demonstrated the ability of NAC to transiently arrest tumor cells in the radiation sensitive G2/M phase of the cell cycle. Using standard clonogenic assays we examined the impact of NAC pretreatment on the cytotoxic activity of the radiomimetic drug Zeocin. Although the unexpectedly high degree of cell death produced by the NAC dose used in these later studies prevented definitive conclusions from being drawn, the results as a whole were encouraging and support further research on the potential of combination therapies employing transient tubulin depolymerizing agents such as NAC and radiation in the treatment of cancer.

Abbreviations Used:

DMEM, Dulbecco's Modified Eagle Medium; NAC, *N*-acetylcholchinol; VDA, vascular disrupting agent; BGS, Bovine Growth Serum; P/S, Penicillin/Streptomycin; DPBS, Dulbecco's Phosphate Buffered Saline; FACS, Fluorescence-activated cell sorter; EtOH, Ethyl Alcohol.

Introduction

Cancer in its myriad forms is one of the largest and most profound health care problems facing the United States. Rectal cancer, as opposed to other cancers of the colon, is specifically limited to the rectum. The cancer has several stages that range from the presence of cancerous polyps (stage 0) to cancer that has fully penetrated the wall of the rectum and invaded surrounding organs and lymph tissue (stage 4). Current established treatment for rectal cancer varies by the stage of the cancer, but follows the pattern of surgery and either chemo or radiation therapy. In a stage 0 cancer the polyps can usually be removed during a colonoscopy. For more severe cancers the treatment involves invasive surgery to remove the affected tissues, and in severe cases to create stoma to replace lost excretory openings in patients were large portions of the rectum and surrounding tissue must be excised. The surgical option is then followed with chemo or radiation therapy and regular testing to determine the effectiveness of treatment and whether more surgery is necessary (National Cancer Institute website).

According to the American Cancer Society rectal cancer has at best a 74% 5 year survival rate (stage 1 cancer) down to a 6% 5 year survival rate (stage 4 cancer). While the drop in survival rate is expected as the cancer spreads, the average over the four stages gives a survival rate of 45 to 50% over five years. Part of the difficulty of rectal cancer treatment resides in the extreme surgery that must be preformed. The complication is twofold. First, the cancer has a high recurrence rate with surgery alone, and thus requires more extensive surgery or a combination of surgery and other therapy[1]. Secondly, the surgery needed to excise the cancerous tissue rapidly becomes very extreme. Any surgery removing large amounts of tissue and fundamentally altering how a system in the human body works is not desirable and can significantly alter patient quality of life[2]. This number leaves significant room for improvement on existing therapy options.

Currently one new option for rectal cancer therapy in development is the use of VDA's or vascular disrupting agents. These drugs target the vascular structure created by the tumor to attack the

tumor by cutting off its blood supply. Without a blood supply the cells in the tumor will not receive the needed nutrients and will die. Combating cancer requires the constant development of new drug therapies. In this light it has been shown that tubulin-depolymerizing agents have significant application in increasing the efficacy of existing cancer therapies. It has been shown that the drug Colchicine causes carcinoma cells to arrest in the G2/M phase of the cell cycle[3]. This alone does not provide a novel concept for therapy. A separate finding showed that the phase of the cell cycle can affect the cell's vulnerability to therapeutic radiation. Specifically, the G2/M phase of the cell cycle shows a significant increase in cell mortality when exposed to therapeutic radiation[4]. These findings suggest that a tubulin-depolymerizing agent could be used to stop the cell cycle at a point where the cell is most vulnerable to radiation. This in turn would cause the same dose of radiation to have a greater effect on a patient’s cancer. However, Colchicine is not a viable agent to use in humans. This is for two reasons. First it is toxic in the doses required, and second its tubulin depolymerizing activity is not readily reversible, which causes an increase in the side effects of the drug[5,6]. If a tubulin depolymerizing agent that had a viable human dose and was rapidly reversible was found it would provide a viable new therapeutic option. The drug NAC is being tested as a possible agent to cause the desired cell cycle arrest.

NAC has been developed as a viable alternative to Colchicine. NAC is the biologically active metabolite derived by de-phosphorylation of the vascular disrupting agent (VDA) ZD6126[7-9]. NAC influences the vascular structure via binding interactions with tubulin[10]. It has been shown that NAC causes a disruption in the vascular structure a tumor for up to 96 hours post exposure to the drug[11]. NAC's effect as a VDA affects the tumor cell in two ways. First it causes the endothelial cells in the tumor vascular system to constrict, and this then causes a decrease in blood supply to the tumor[12]. This drop in blood supply alone can cause adverse effects to the tumor[13]. Secondly, as mentioned above, it has been noted that the use of NAC causes a reversible pause in the cell cycle at the G2/M phase that is

especially noticeable in rapidly dividing cells, such as the endothelial cells in a tumor[14]. It is also important to not that NAC does not exhibit the toxic effects that make Colchicine difficult to use in humans[15].

Combining this knowledge, the object of our study is to determine the effect that the use of NAC can have on the existing effectiveness of therapeutic radiation. The purpose is to show that the cell cycle pause caused by exposure to NAC can then be combined with existing therapeutic radiation to create a situation where the same dose of radiation would have a significantly greater effect on tumor cell mortality, and would likely then show an increase in patient outcomes and quality of life recovering from rectal cancer[16]. If VDAs such as NAC can be implemented in patients in an effective dose treatment options will gain effectiveness or maintain the same effect but show a reduction in side effects.

Due to the intrinsic hazards of radiation exposure the antibiotic Zeocin was used in place of radiation exposure. Zeocin is a radiomimetic antibiotic from the bleomycin/phleomycin family of antibiotics[17]. Zeocin acts on the genetic material of the cell by inducing double strand DNA breaks[18]. In healthy cells the presence of Zeocin can induce an increase in p53 production and, if the dose is high enough, apoptosis[19,20]. Zeocin is a strong enough actor on the genetic material of the cells it enters that even cells given resistance to the antibiotic are still subject to significant stress from induced double strand breaks[21]. It has been suggested that cells that have greater ability to rejoin broken DNA are more able to survive Zeocin exposure, but they still suffer the same damage[22]. These DNA breaks are enough to cause significant cell mortality in cells that do not have specific ability to resist the Zeocin.

The study will focus on the cell line designated CMT-93. CMT-93 is a murine epithelial rectal carcinoma. The CMT-93 cells are adherent and thus were grown attached in Falcon cell culture flasks. The cells were grown in medium containing DMEM along with BGS and P/S antibiotics. The BGS provided necessary nutrients for cell growth and the P/S antibiotics helped prevent bacterial

contamination. The cells were grown at human physiological temperature and carbon dioxide concentration. The primary method of study was clonogenic assays. These are studies where cells are exposed to some experimental factor and then plated in known quantity onto growth plates. The cells are then allowed to grow for about one week. This allows any cells that survived the experimental protocol healthy enough to reproduce to establish colonies that will be visible after staining with dye.

First the CMT-93 line was subjected to a plating efficiency test. This involves plating a known quantity of cells to determine how many unique colonies can be obtained. This gives a rough percentage of cells plated that will survive the transfer process and begin growing in a new container. This is essential because later experiments will require knowledge of how many cells must be plated to attain certain numbers of surviving colonies. For our purposes the protein binding dye Methylene Blue will be used to stain the colonies.

The second phase of the experiment was to expose the CMT-93 cell line to a variety of concentrations of NAC and Zeocin. The cells were exposed to a range of concentrations of the two drugs independently to establish the effect different doses have on the ability of the cells to survive. This was determined using the clonogenic assay described above. The goal was to find a dose of NAC that does not have a significant toxic effect and a dose of Zeocin that has some toxic effect. This established suitable doses of the drugs to use when studying them in combination.

Following the establishment of a suitable dose of NAC the CMT-93 cells were exposed to NAC and harvested at several time points after the drug exposure. These collected cells were be fixed in ethanol and subjected to FACS analysis. FACS uses a florescence-inducing laser to detect the presence of cells stained with some sort of florescent marker. Typically this is used to sort separate types of cells as a purification method[23]. For our purposes the FACS was used to detect the level of florescence to determine cell cycle distribution. The level of florescence will vary directly with amount of stained genetic material in a given cell. This analysis will be used to measure the amount of genetic material

present in the cells and characterize the kinetics of the cell cycle arrest. The cells were stained with Propidium iodide immediately before analysis[24]. This analysis of the quantity of genetic material present against the size of the cell will allow a characterization of the distribution the cell cycle phase of the cells in the sample. Cells in the G2/M phase of the cell cycle appear with twice as much brightness per unit of size as cells in the G1 phase. The data achieved this way showed what period of time elapses before NAC induces the mentioned cell cycle pause. The time determined through FACS analysis provided the final data required to study the drugs in combination.

The final phase of the experiment was to study the effect that a pre-dose of NAC has on the efficacy of Zeocin. This was preformed by dosing cells with NAC and waiting for the time point established by FACS analysis. At this time point the cells were dosed with Zeocin. The cells were then plated into new dishes and allowed to grow. Samples were also dosed with only NAC, only Zeocin, and no drugs to serve as control samples. In this experiment and the preceding characterization studies cells were be counted by staining with Methylene Blue and use of ColCount software and counting machine. We hypothesized that this experiment would show that Zeocin has increased efficacy when used following NAC.

Due to the sensitive nature of the cells, the drug studies, and the goals of the research, all of the work was done under sterile conditions. All work was be done inside a sterile biological cabinet. Any materials used were either be sterilized through an autoclave or pre-sterilized from the manufacturer. Any sterile packaging was be opened in the sterile cabinet. This hopefully prevented any contamination of the cell line and alteration of the results. If cell stock was found to be contaminated it was disposed of and new cell stock was be thawed.

Materials and Methods:

Cell Line:

The murine rectal carcinoma line CMT-93 was obtained from the American Type Culture Collection and was thawed from stock pre-frozen at -80^{o}C. These cells were thawed in a 37^{o}C water bath. The cells were cultured in a mix of DMEM, BGS, and P/S antibiotic. Cells were split every 2 to 3 days. To split into a new flask the cells must be harvested. This was done by washing the cells 3 times with DBPS by pouring off the growth media and pouring DPBS into the flask. After three washes, about 3 mL trypsin was added to the flask (enough to make a monolayer over the cells). This was then allowed to sit for approximately 5 minutes. Cells are then removed from the flask by a 5 mL pipette and diluted up to 10 mL with growth media. About 0.5 mL of this mix is placed into 30 mL fresh growth media in a new flask. This was done as needed to maintain cell stock throughout the experiment. Multiple flasks were grown as needed to provide sufficient cell numbers for experimentation. Cell stocks were grown in an incubator at 37^{0}C and atmosphere containing 5% CO_2.

Plating Efficiency:

CMT-93 cells were harvest as described above. The concentration of cells in the media after harvesting was determined by counting the cells with a Hemocytometer. Based on this number a solution of 10 mL media at 1000 cells/mL was made in a 15 mL tube. 5 mL of this solution was then mixed with 5 mL media to make 10 mL media at 500 cells/mL. This series of 1 to 1 dilutions was repeated several times to make a final series of cell concentrations of 1000., 500.0, 250.0, 125.0, 62.5, 31.25, and 15.13 cells/mL. 1 mL of each of these solutions was added to each of three 10 cm growth dishes. These dishes were then allowed to grow for about 1 week. They were then washed by pouring off the media and pouring on DBPS three times. The last DBPS was poured off and 4 mL of Methylene Blue was pipetted onto the cells. This was allowed to stand for 30 minutes to 1 hour. The Methylene

Blue was then pipetted back into the stock and the cells were washed in purified water to remove excess dye. The cell colonies were then counted using ColCount software and equipment.

NAC Dose Response:

CMT-93 cells were exposed to several different NAC concentrations to discern a nontoxic dose of NAC. NAC stock at 100 mg/mL was used. This stock was diluted to make concentrations of 200., 100., 50.0, 25.0, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.18 ug/mL. CMT-93 cells were exposed to each of the above concentration in suspension for 30 minutes at 37^{o}C in a water bath. There was also a no drug exposure control run. These were plated at 500 cells per plate onto three 10 cm plates per drug dose. The cells counts were determined using a Hemocytometer as above. After being allowed to grow for about one week the cells were stained with Methylene Blue and counted using ColCount. The experiment was also run using NAC concentrations of 200., 100., 50.0, 25.0, 12.5, 6.25, 3.13, and 0 ug/mL and plating 4000 cells per plate.

Transient and Continuous Zeocin Dose Response:

The effect Zeocin on CMT-93 cells was to be characterized in both transient and continuous exposure. For the transient experiment 24 well plates were used. Each well on a 24 well plate was plated with 10^5 cells. Stock cells were harvested and counted with the Hemocytometer to determine appropriate volumes. These were allowed to attach overnight. Starting from 100 mg/mL stock Zeocin, 3 mL of 4000 ug/mL was made. 1.5 mL of this was added to 1.5 mL media. Through several of these 1 to 1 dilutions solutions of 4000., 2000., 1000., 500.0, 250.0, 125.0, 62.5, 31.25, 15.63, and 7.81 ug/mL were made. From each of these 0.5 mL was added to 0.5 mL media in two wells of the 24 well plate for final concentrations of 2000., 1000., 500.0, 250.0, 125.0, 62.5, 31.25, 15.63, 7.81, and 3.91 ug/mL. Two wells were also exposed only to media. The cells were exposed to Zeocin for 30 minutes at 37^{o}C

and 5% CO_2. The drug solution was suctioned off. Each well was then washed three times with 0.5mL DBPS. The DPBS was removed using suction. 0.5 mL media was then placed in each well. The cells were left to sit overnight. The next day all the wells were harvested. This was done with 0.5 mL trypsin per well. The harvested cells were brought up to 5 mL media and counted using a Hemocytometer. The cells were then plated at either 1000 cells per 10 cm plate for three plates per dose, or at 4000 cells per plate at three plates per dose as the experiment was preformed twice. After one week of growth the plates were stained with Methylene Blue and counted using ColCount. The Zeocin characterization was also done under continuous exposure conditions. For this Zeocin solutions were made at concentrations of 1000., 500.0, 250.0, 125.0, 62.5, 31.25, 15.63, 7.81, and 3.91 ug/mL with a volume of 20 mL per concentration. Cells were the harvested from cell stock and counted using the Hemocytometer. For each dose 1000 cells were plated into 10 mL of the Zeocin solutions in each of two 10 cm plates. These were allowed to grow for one week. After one week all the plates were stained with Methylene Blue and counted using ColCount.

FACS Cell Cycle Analysis:

FACS analysis was done to observe the kinetics of the cell cycle arrest caused by NAC exposure. 4 flasks of cells were harvested into a 50mL centrifuge tube and brought to a total volume of 33.5 mL with media. 3.5 mL were removed for later use. The tube was spun down at 1000 rpm and 20^{o}C for 10 minutes. The supernatant was poured off and the cells were resuspended in 20mL media. 10 mL of this was transferred to a second 50 mL tube. Both of these tubes were spun down under the same conditions as the first tube. The supernatant was poured off each tube. One tube was resuspended in 10 mL media and the other in 10 mL of 100 ug/mL NAC. These were incubated in a 37^{o}C water bath for 30 minutes. While the cells were incubating the 3.5 mL removed at the start was brought up to 25 mL with DPBS and spun down at 1000 rpm and 20^{o}C for 10 minutes. After pouring off the supernatant,

the cells were resuspended in 70% EtOH as a zero time point control sample. After 30 minutes the incubating tubes were spun down at 1000 rpm and 20^{o}C for 10 minutes. The supernatant was poured off and the cells were resuspended in 10 mL media to wash off remaining NAC and then spun down again under the same conditions. This was repeated three times. Following this each tube of cells was resuspended in 40 mL of media. 10 mL was plated into each of four 10 cm plates for each tube. At time points of 6, 12, 24, and 48 hours post-drugging, 1 plate of drugged and 1 plate of nondrugged cells were harvested using 3 mL of trypsin. The cells were harvested into separate 50 mL centrifuge tubes and brought up to 25 mL with DPBS. They were then spun at 1000 rpm and 20^{o}C for 10 minutes. The supernatant was poured off and each sample was resuspended in 5 mL of 70% EtOH. All EtOH suspension tubes were stored in a 4^{o}C refrigerator. On the day of the FACS analysis all the EtOH suspension tubes were spun at 1000 rpm and 20^{o}C for 10 minutes. The samples were then resuspended in 5 mL of 500 ug/mL Propidium Iodide and 100 ug/mL RNaseA in DPBS. They were allowed to stain in this solution overnight in foil wrapped tubes. The cells were then submitted for FACS cell cycle analysis. The experiment was also repeated using 50 ug/mL NAC as the only procedural difference.

Zeocin-NAC combination Study:

Two flasks of CMT-93 were harvested and brought up to 10 mL with media in a 50 mL centrifuge tube. The cells were counted using a Hemocytometer and plated into each well of a 24 well plate and a concentration of 10^5 cells per well. These cells were allowed to attach overnight in 0.5 mL media. Following this 0.5 mL of 100 ug/mL NAC was added to the 0.5 mL media in two of the four rows of wells on the plate. This dosed half the cells with 50 ug/mL NAC. The other half only had 0.5 mL media added. The cells were left to incubate in a 37^{o}C and 5% CO_2 incubator for 30 minutes. After this the media was suctioned off and the cells were washed three times with DPBS. After washing 0.5 mL media was placed in each well an the plate was incubated until the 24 hr cell cycle arrest induced

by NAC. At the 24 hr point one row of NAC exposed wells and one row of non-NAC exposed wells had 0.5 mL of 250 ug/mL Zeocin added to their 0.5 mL media for a 125 ug/mL dosing concentration. The other wells received 0.5 mL media. This was allowed to incubate for 30 minutes. The media and drug was then suctioned off and the cells were washed three times with 0.5 mL DPBS and placed in 0.5 mL media to sit overnight. This produced one row of wells of each of the following: NAC and Zeocin exposure, only NAC exposure, only Zeocin exposure, and only media exposure. The wells were harvested using 0.5 mL trypsin and like wells were placed in the same 15 mL centrifuge tubes. These cells were counted using a Hemocytometer and plated at 1000 cells per plate onto each of six 10 cm plates for each drug dose. These plates were allowed to grow for one week. The plates were stained with Methylene Blue and counted using ColCount.

Results:

The first section of the experiment was the plating efficiency test on CMT-93. Three 10 cm plates were tested for cells counts of 1000, 500, 250, 125, 62.5, 31.25, and 15.13 cells per plate. After all three plates were counted the mean number of colonies obtained was 187, 87.67, 49.33, 24.67, 11.33, 7.00, and 3.00 respectively. These numbers are graphed in Figure 1 to show the smooth progression of colonies obtained versus cells plated.

Following this the effect of NAC on the survivability of the CMT-93 line was determined. This experiment was run twice. The first used NAC doses of 200., 100., 50.0, 25.0, 12.5, 6.25, 3.13, and 0 ug/mL as a control. After counting three plates for each dose at 4000 cells per plate the mean surviving colonies numbered 969, 1077, 1134.33, 960.67, 1021, 1156.67, 1055, and 1000 respectively. This data is plotted in Figure 2. The experiment was run a second time plating 500 cells per plate and using drug doses of 200., 100., 50.0, 25.0, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.18, and 0 ug/mL NAC. The mean colonies obtained over three plates was 137.33, 145.33, 121.67, 118, 132.67, 67.67, 81.33, 62, 77.67,

83.67, 59.33, and 107.33 respectively. This data set can be seen plotted in Figure 3.

Next the effect of Zeocin on the CMT-93 cells was determined. The first experiment was done using Zeocin concentrations of 2000., 1000., 500.0, 250.0, 125.0, 62.5, 31.25, 15.63, 7.81, 3.91, and 0 ug/mL. The mean colonies obtained from about 4000 cells per plate were 349.67, 565.67, 875.33, 898, 996.33, 1080.67, 1101.33, 1070.67, 1011, 1013.33, and 1197.67. This data and an expanded view of the data set ranging from Zeocin doses 0 to 500 ug/mL can be found in Figure 4. The second run of this experiment was done using the same set of Zeocin concentrations but plating 1000 cells per plate. The mean colonies per plate were calculated to be 59.33, 136, 262, 307.33, 223.67, 98.67, 101.67, 118.33, 70.33, 78.67, and 309.33 respectively. This experiment was done plating 1000 cells per plate, and the data can be seen in Figure 5. After this transient exposure test a continuous Zeocin exposure assay was preformed. Two plates of approximately 1000 cells per plate were allowed to grow under constant Zeocin exposure of 1000., 500.0, 250.0, 125.0, 62.5, 31.25, 15.53, 7.81, 3.91, and 0 ug/mL Zeocin for one week. The mean colonies obtained respective to drug concentration were 0, 0, 0, .5, 19.5, 102, 196.5, 231, 273.5, and 316.5. The continuous Zeocin exposure data in plotted in Figure 6.

After the drug characterizations were complete the kinetics of the NAC induced cell cycle arrest were analyzed via FACS analysis. The FACS data for the time point at 12 hours after drugging with 100 ug/mL NAC is seen in Figure 7. The red peak at DNA content ~80 shows the proportion of the cells in G2/M phase and the small red peak at ~50 shows G1 phase cells. This experiment was repeated using 50 ug/mL NAC. The data for the time point 24 hours post 50 ug/mL exposure is in Figure 8. Two peaks, yellow at DNA content ~80 and red at ~50, show the proportion of cells in the G2/M and G1 phases respectively. Figure 9 shows the data for 48 hours post 50 ug/mL NAC drugging. The yellow peak at DNA content ~160 indicating polyploidy was unexpected.

The final phase of the experiment was to determine the effect of the drugs in combination. Six plates at 1000 cells per plate were made for exposures of media only, 50 ug/mL NAC, 125 ug/mL

Zeocin, and 50 ug/mL NAC followed by 125 ug/mL Zeocin. The respective means of the colonies obtained were 232.5, 21.17, 125, 17.67. This data can be seen plotted in Figure 10.

Discussion:

There were several steps required to find the characteristics of the cells and the effects the drugs had on the CMT-93 cells. The first phase of this was to determine the plating efficiency of the CMT-93 cell line. This is done so that a baseline survival rate can be established for the cells. This provides both a beginning knowledge of simply how many cells must be plated to attain sufficient numbers of surviving colonies for study, and also shows, through this baseline, what proportion of cell loss is due to the transfer from one container to another. It is important to know this so that the loss of colonies compared to cells plated is not incorrectly attributed to effects of the drugs involved in the study. The plating efficiency data obtained suggested that plating 1000 cells would likely yield 187 surviving colonies with a standard deviation of 7. This posits 1000 cells as a good number to plate. Obtaining 200 colonies does not overcrowd the plates, but still is enough colonies that significant changes in number from experimentation can be observed. It is also key to realize that while this provides basic information it is not law. It is important to know that controls will be done for every study preformed so that the trend can be observed. Having an idea of the cell plating efficiency does not allow us to perfectly predict the number of colonies that will be obtained every experiment.

Following the establishment of the plating efficiency the effect of NAC was determined on the CMT-93 through the clonogenic assay. The first run of the experiment saw a successful drugging, but an error was made in the plating of the cells. The cells were plated onto their 10 cm growth dishes at close to 4000 cells per plate. This is problematic because at that concentration other factors enter into the survival of the cells. Specifically, overcrowding and media starvation become issues that will alter the experimental data. The data covers a range of NAC doses from the low (3.13 ug/mL) to the high

(200 ug/mL) The results show in Figure 2 seem to be sound, but because of the number of cells plated they cannot be expected to stand alone. They do, however, suggest that only high doses of NAC have a significant cell mortality rate. As a result of the cell plating error the experiment was run a second time with a target of 500 cells per plate. The experiment was done with the same range of NAC doses and the addition of doses 1.56, 0.78, 0.39, and 0.18 ug/mL to determine the effect of very low drug concentration. The data from this experiment is more valid due to the lower number of cells plated. As seen in Figure 3, the data shows that, barring some inconsistency, the cells survive well even at high doses of NAC. The control sample had a standard deviation of 22.48. Some of the higher NAC doses show a higher number of surviving colonies than the control sample. The data at 200.0, 100.0, and 12.5 ug/mL is higher than the standard deviation of the control. This suggests that there might be some effect of the NAC enhancing cell survival. Another possibility is that there were again errors in the number of cells plated and some of the drug doses received more cells. It is important to find a dose of NAC that does not have a toxic effect on the cells. Because the purpose of the experiment is to determine if NAC increases the efficacy of other therapeutic options, the NAC itself cannot be responsible for cell mortality.

The next phase was to characterize the effect that Zeocin had on the cell line. This must be done to find proper doses for study in combination with NAC. The transient Zeocin exposure was, as with NAC, done twice. The first experiment with Zeocin suffered the same problem as the first NAC experiment. Instead of cells being plated at the desired amount, they were plated at around 4000 cells per plate. This again causes the problems in determining the origin of any cell mortality. The data for Zeocin doses from 2000 ug/mL down to 3.91 ug/mL show that there is toxicity as compared to the no drug control sample, but the data cannot really stand alone because of the potential overcrowding in the plates with such high cell numbers. Figure 4 shows this trend. The experiment was repeated with a desired plating of about 1000 cells per plate. This did not come to pass. The dose range of Zeocin

shows that high doses cause significant mortality, but at low Zeocin doses there were very few colonies obtained. This is most likely due to plating error. The same volume of each solution of cells was plated. Because the volume of cell solution plated of each drug sample was not determined individually, but was the same across all samples error was introduced. The samples at low drug concentration seem to have had fewer cells/mL and thus had significantly fewer cells plated. This causes the dip in colony numbers observed in Figure 5. The trend in Figure 5 lends enough credibility to the data from Figure 4, however, that we can assume a Zeocin dose in the area of 125 ug/mL will be sufficient. It causes some cell mortality, but there are still significant numbers of colonies obtained. The number of colonies obtained allows visualization of an increase in efficacy of the same dose. Continuous Zeocin exposure was also studied. This was done to further understand the effect of Zeocin on the cell line and to observe what Zeocin that was not successfully washed off of cells could do to results. The cells were grown for one week under constant Zeocin exposure in concentrations from 1000 ug/mL to 3.19 ug/mL with a control sample. The data, as seen in Figure 6, suggests that constant Zeocin exposure has a drastic effect on cell mortality. The three highest doses had a 100% mortality rate and only one colony was obtained from the plates at 125 ug/mL Zeocin. The lowest doses of Zeocin showed similar effects on cell mortality to their effect in the transient study. These results underscore the importance of washing the drugging solutions off of the cells after the experimental procedures. Even small amounts of drug remaining on the cells while they grow can further alter the ability of the cells to survive. If this happened during the combination experiment it would cause a false increase in the efficacy of Zeocin from the NAC.

Following the determination of the effects of Zeocin on the CMT-93, the kinetics of the cell cycle arrest caused by the NAC were determined. This was done by FACS analysis. This allows a quantification of DNA present in the cells due to staining with florescent dye. The FACS machine uses a laser to induce this florescence and can analyze the cells accordingly. For our purposes the amount of

DNA in each cell will indicate what part of the cell cycle the cell is in. The cell sample will have populations at both the G1 phase with one level of florescence and the G2/M phase with twice the brightness of the G1 cells. The time point where NAC induced arrest occurs will be the point at which the vast majority of the cells are in the G2/M phase. After preparation and staining with propidium iodide the samples dosed with 100 ug/mL were analyzed. The data from this study, as seen in Figure 7, show that the vast majority of the cells at the 12 hour time point (the large yellow peak at ~80 on the DNA content axis) are in the G2/M phase. The small red peak at ~50 on the DNA content axis is the proportion of cells in the G1 phase. The problem is that there was significant cell death in the 48 hour time point. The mortality was observed in the sample prior to FACS analysis. As a result of this mortality the experiment was redone using 50 ug/mL NAC. These samples were prepared in an identical manner and the samples seemed healthy at the time of preparation. At this dose of NAC the cell cycle arrest is delayed to the 24 hour time point. This is seen in Figure 8 as the large yellow peak at ~80 on the DNA content axis compared to the much smaller red peak at ~50. This delay in the arrest is likely only due to the lower drug dose taking a longer time to act. The oddity in these results comes from the data in Figure 9. This shows a significant portion of the sample still in G2/M arrest, but there is also a large polyploid population shown by the peak at ~160 on the DNA content axis. This seems to suggest that the cells have gone through a second DNA replication without dividing and now have four times the normal genetic material. The obvious explanation would be that clumps of cells are being read as on cell and this polyploidy is a result from that. Because the FACS machine sorts cells by size, the FACS machine only counts individual cells. Clumps of cells are not counted in the data set. The second explanation is that the NAC has a more profound effect on the CMT-93 cells. This could come from the drug being less reversible than thought. This would in turn cause the tubulin to stay depolymerized and prevent mitosis. It is then conceivable that the cell could attempt to divide again given that the normal apoptotic safeguards are disabled in the cancerous cells. The other explanation is

that the NAC was not sufficiently washed off the cells and the continued presence of the drug caused the cells to be unable to divide but able to repeatedly replicate their DNA.

The final phase of the experiment was to run a combination study of the NAC and Zeocin. As described above, this was done using 50 ug/mL NAC and 125 ug/mL Zeocin. The control sample showed a mean colony count of 232.5 over all six plates. As expected, the plates only receiving dosing with 125 ug/mL Zeocin show some cell death but not total loss with a mean of 125 colonies obtained. Finally, the combination sample showed a mean of 17.67 colonies over all six plates. This showed that the combination of the two drugs had very high mortality rate. The problem arises in that the plates exposed only to 50 ug/mL NAC only gave a colony count mean of 21.17 with a standard deviation of 15.01. This shows that the NAC had a significant toxic effect. Further, this essentially negates the result in the combination plate because there is no way to be sure that the mortality observed is a result of the Zeocin. If the NAC itself is causing the mortality in the cell populations it is not working as predicted.

The even more problematic facet of this result is that it is very difficult to explain. The lack of an error across all samples in the combination study lowers the likelihood of a gross procedural error. This is also supported by the results from the control and Zeocin only samples performing as expected. There is also support for the NAC causing toxicity in the results from the FACS data. Recall that the data from the 48 hour time point on the FACS analysis of 50 ug/mL NAC showed unexplained ploy ploidy. This anomalous result also suggests some aspect of NAC that was not expected. Despite this, there is support for the NAC not being at fault. In both of the NAC dose response studies done on the CMT-93, the NAC did not show significant toxicity at the dose of 50 ug/mL. In fact, in both those data sets the 50 ug/mL NAC exposed plates had more colonies than the control plates that had no drug exposure. While the fact that they had more colonies is likely a chance error, the key result is that twice the NAC showed no toxicity at this dose. There is one strong possibility to explain this error. It is possible that the NAC used in the dose characterizations was degraded or that the NAC in the

combination and FACS studies was a more active batch. If either of these was the case it would result in the doses used in the later experiments having greater toxicity than expected.

Regardless of the above results, there is potential for the NAC to still be a successful therapy. While this result does not indicate that the combination of NAC and Zeocin is effective in the manner predicted, it also does not answer all questions. There is still the unexplained toxicity from the NAC seen in the final experiment, and the unusual polyploidy seen in the FACS analysis. The contradiction between the sets of results regarding NAC suggest that a further characterization of the drugs effect, specifically on CMT-93, is merited. This will determine if the results noted above come from some error in the execution of the procedure, or a novel facet of the NAC.

Figure Legends:

Figure 1: The graph shows the plating efficiency of CMT-93 on a plot of Colonies Obtained versus cells plated. A clear correlation is seen between more plated cells and more colonies obtained.

Figure 2: Shown in this figure is the dose response of CMT-93 to NAC on a plot of Colonies Obtained versus NAC dose in ug/mL. The plot shows that there is not high toxicity in the NAC doses tested, however the number of cells plated was larger than allowed for a reliable measurement.

Figure 3: This figure shows Colonies Obtained versus NAC dose in ug/mL for 500 cells plated. The data shows that doses in the 50 to 100 ug/mL range do not have high toxicity in CMT-93 cells.

Figure 4: The plot shows the first of the Zeocin dose response studies on a plot of Colonies Obtained versus Zeocin dose in ug/mL. The plot shows high toxicity in the higher doses, but the number of cells plated was larger than allowed for a reliable measurement. The Lower plot is a expansion of the doses from 0 to 500 ug/mL for clarity.

Figure 5: Shown here is the plot of the second Zeocin dose response. It is thought that the data associated with the dose concentration from 3.91 to 250 ug/mL is compromised by a cell plating error.

Figure 6: The Zeocin continuous exposure assay is shown in this figure. The plot shows a clear relation between presence of Zeocin and inability of cells to grow. No dose above 62.5 ug/mL had more than one total colony survive.

Figure 7: This figure shows the FACS analysis for the time point 12 hours after the addition of 100 ug/mL NAC. The large yellow peak shows cells in the G2/M phase while the small red peak shows cells in the G1 phase. The high portion in G2/M indicates that the NAC induced cell cycle arrest is occurring.

Figure 8: This chart is the 24 hour drugged time point for the 50 ug/mL NAC exposure FACS analysis. The large yellow peak shows cells in G2/M and the small red peak shows cells in G1. As in Figure 7 the high portion of cells in G2/M shows that NAC induced cell cycle arrest.

Figure 9: This shows the presence of polyploidy in the 48 hour drugged time point of the 50 ug/mL NAC exposure FACS analysis. The red peak indicated G1 phase cells, the yellow peak at DNA content 80 is cells in the G2/M phase, and the yellow peak at DNA content 160 is a population polyploid cells.

Figure 10: The final chart shows the Colonies Obtained versus Drug dose for the combination study. The columns show unexplained toxicity in the NAC sample that calls the result on the NAC and Zeocin exposed sample into question.

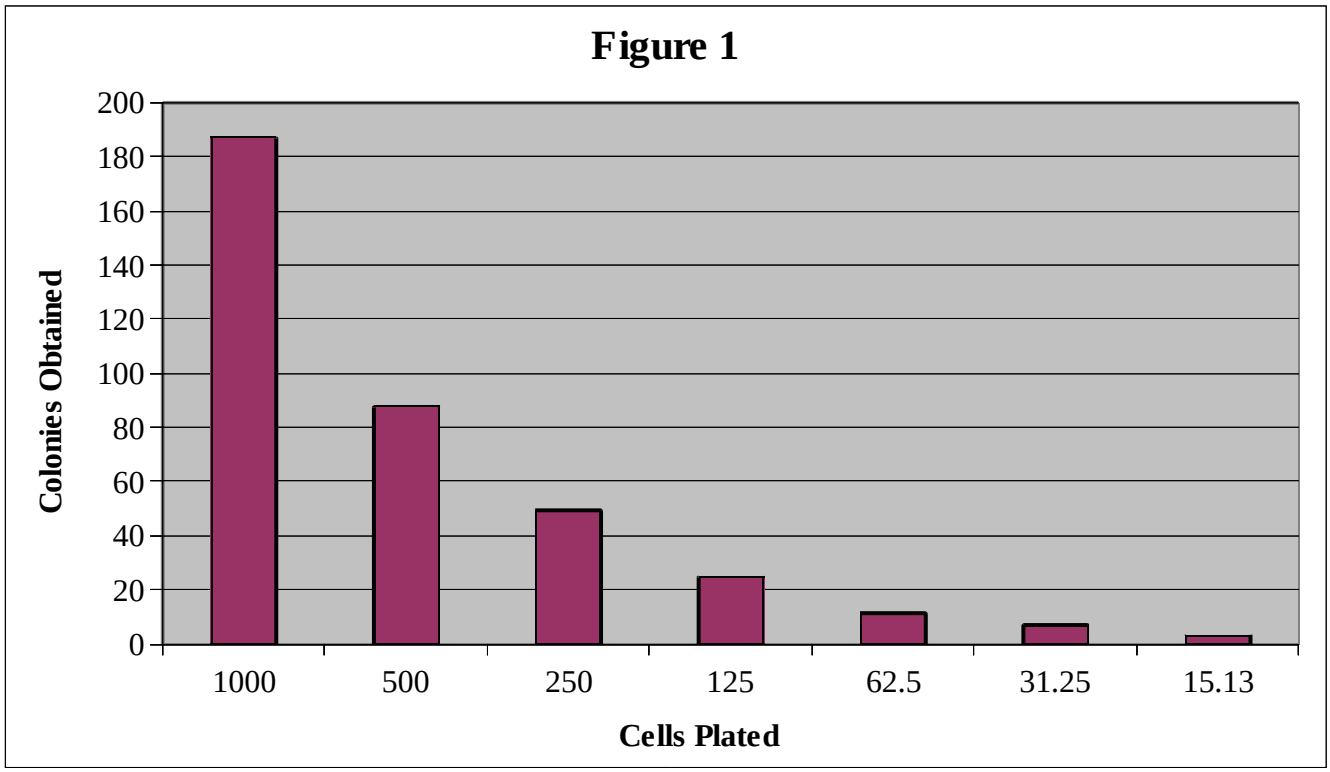


Figure 1
Colonies Obtained
200
180
160
140
120
100
80
60
40
20
0
1000
500
250
125
62.5
31.25
15.13
Cells Plated

Figure 2

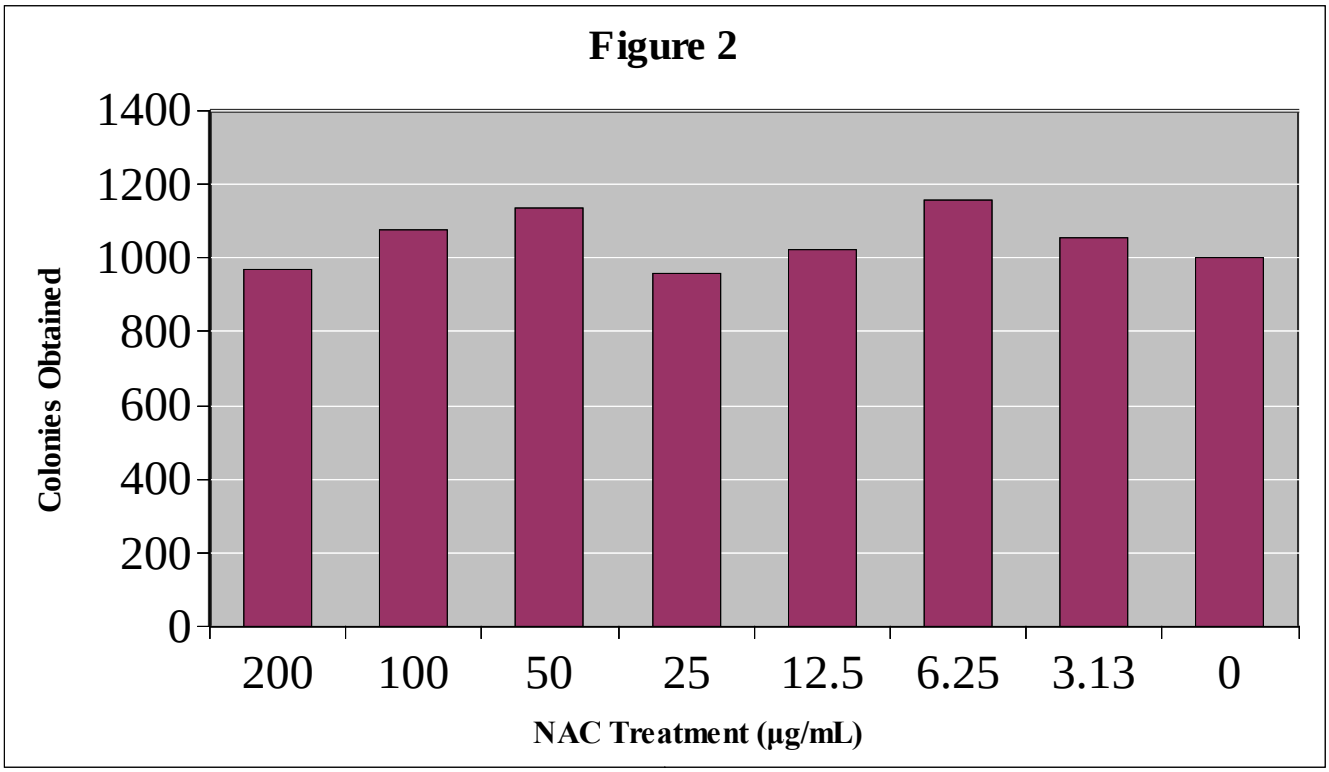


Figure 3

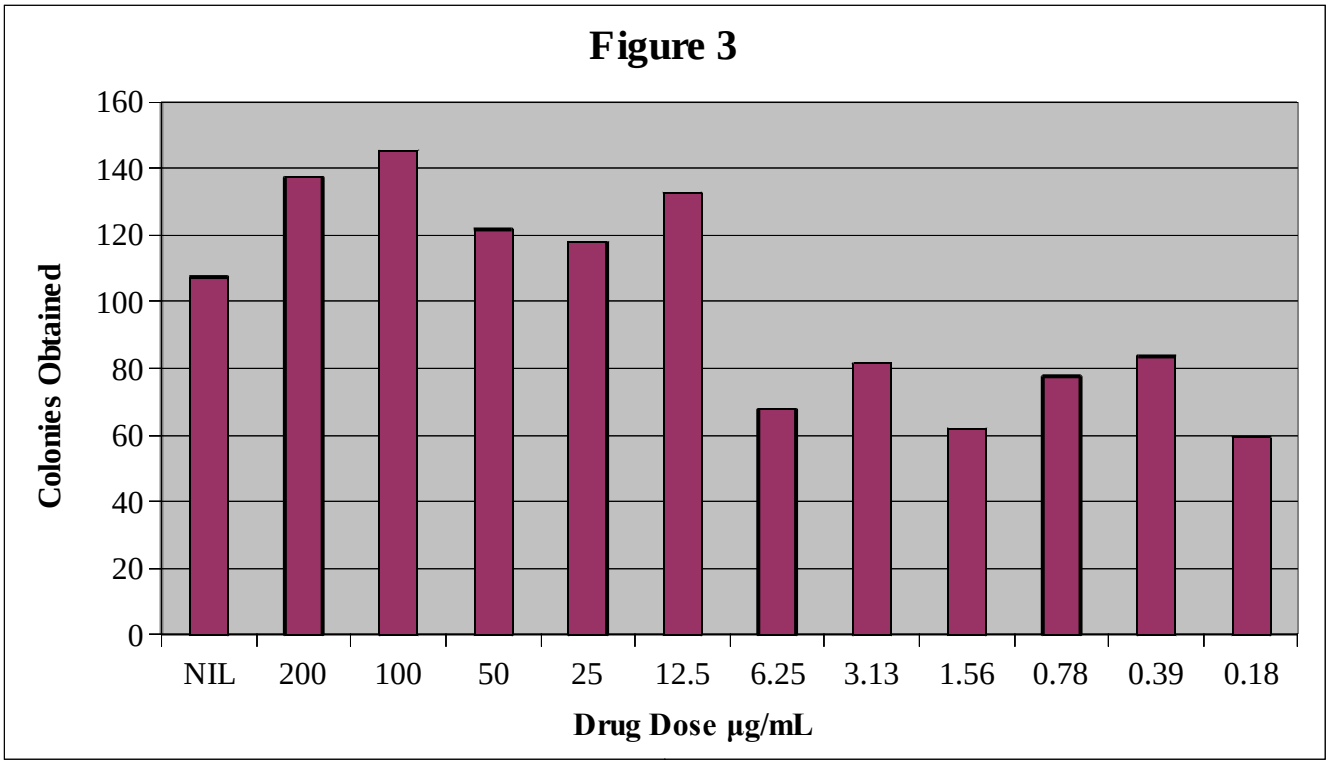


Figure 4a

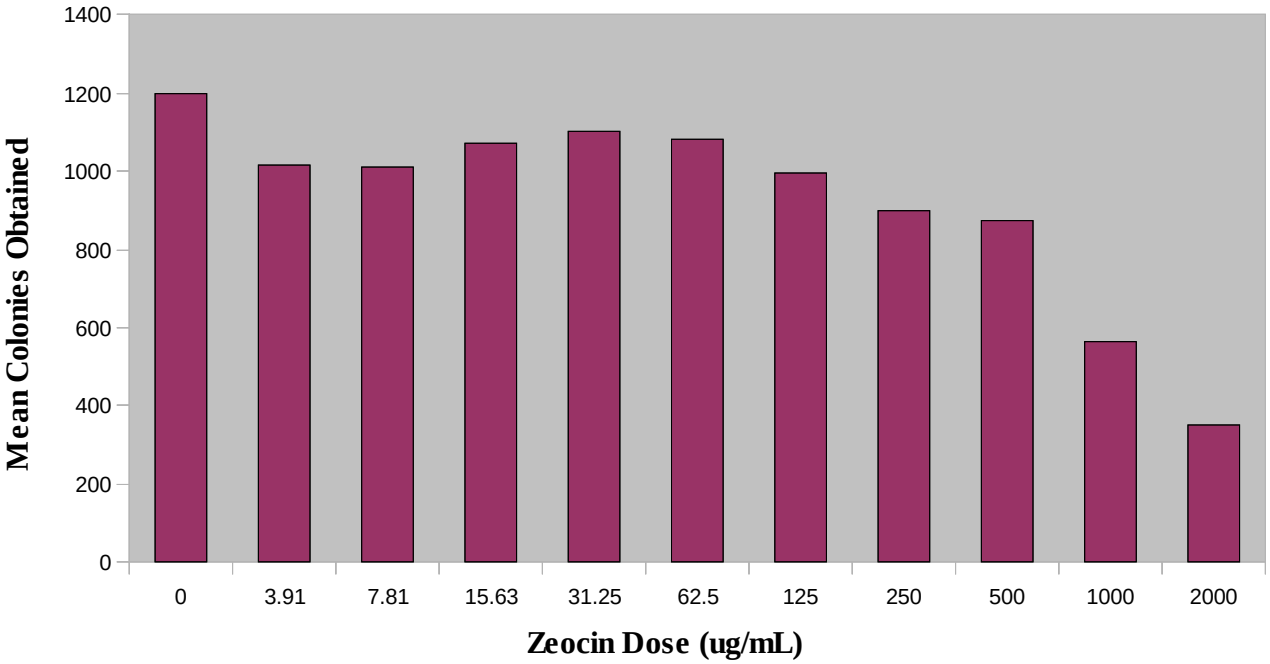


Figure 4b

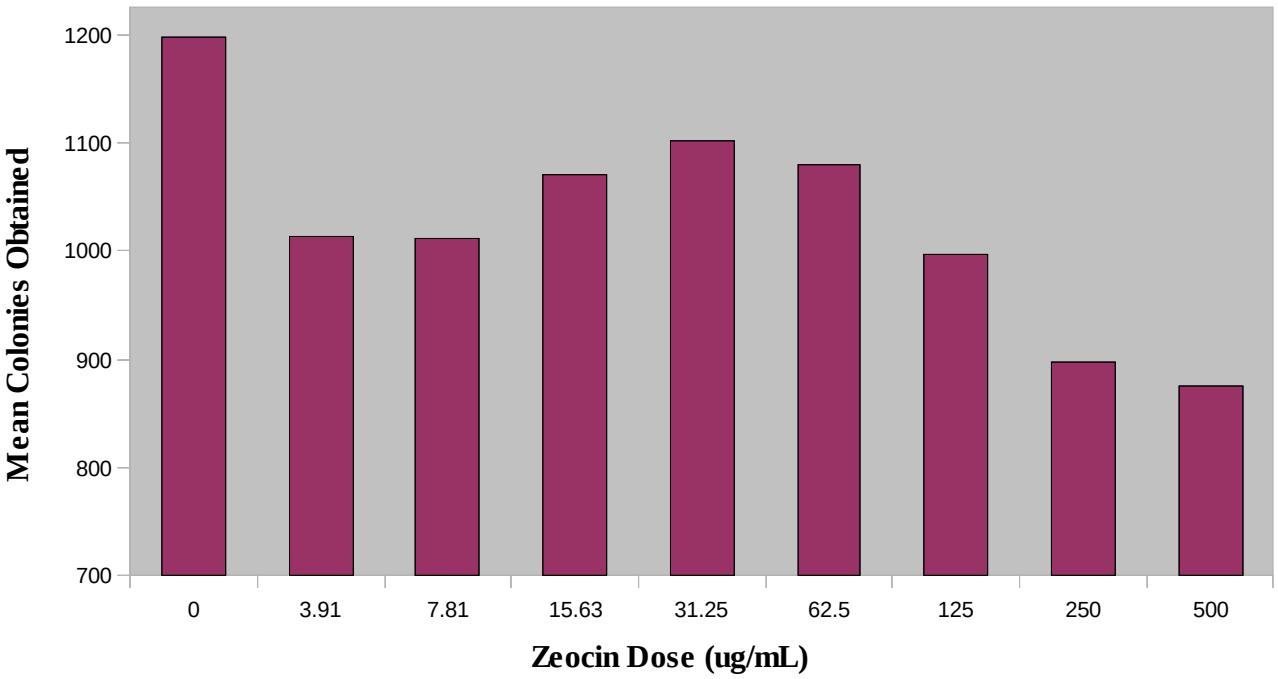


Figure 5

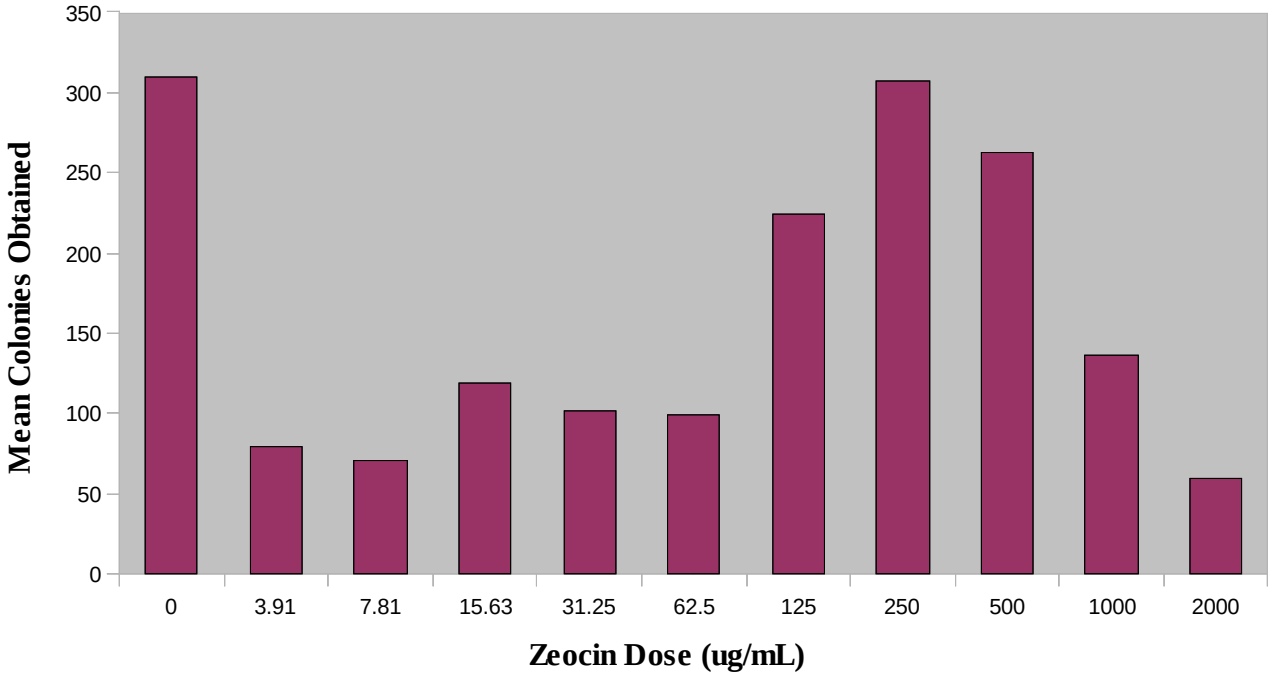


Figure 6

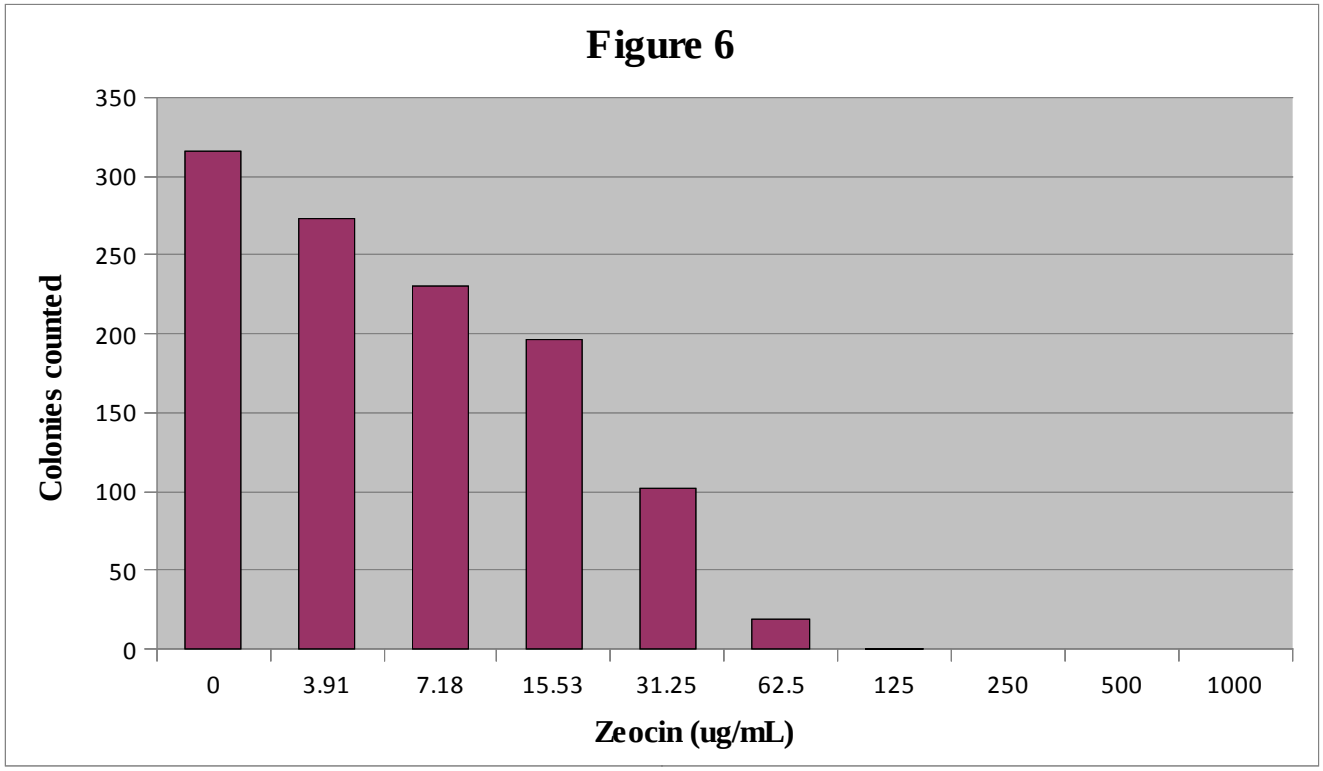


Figure 7

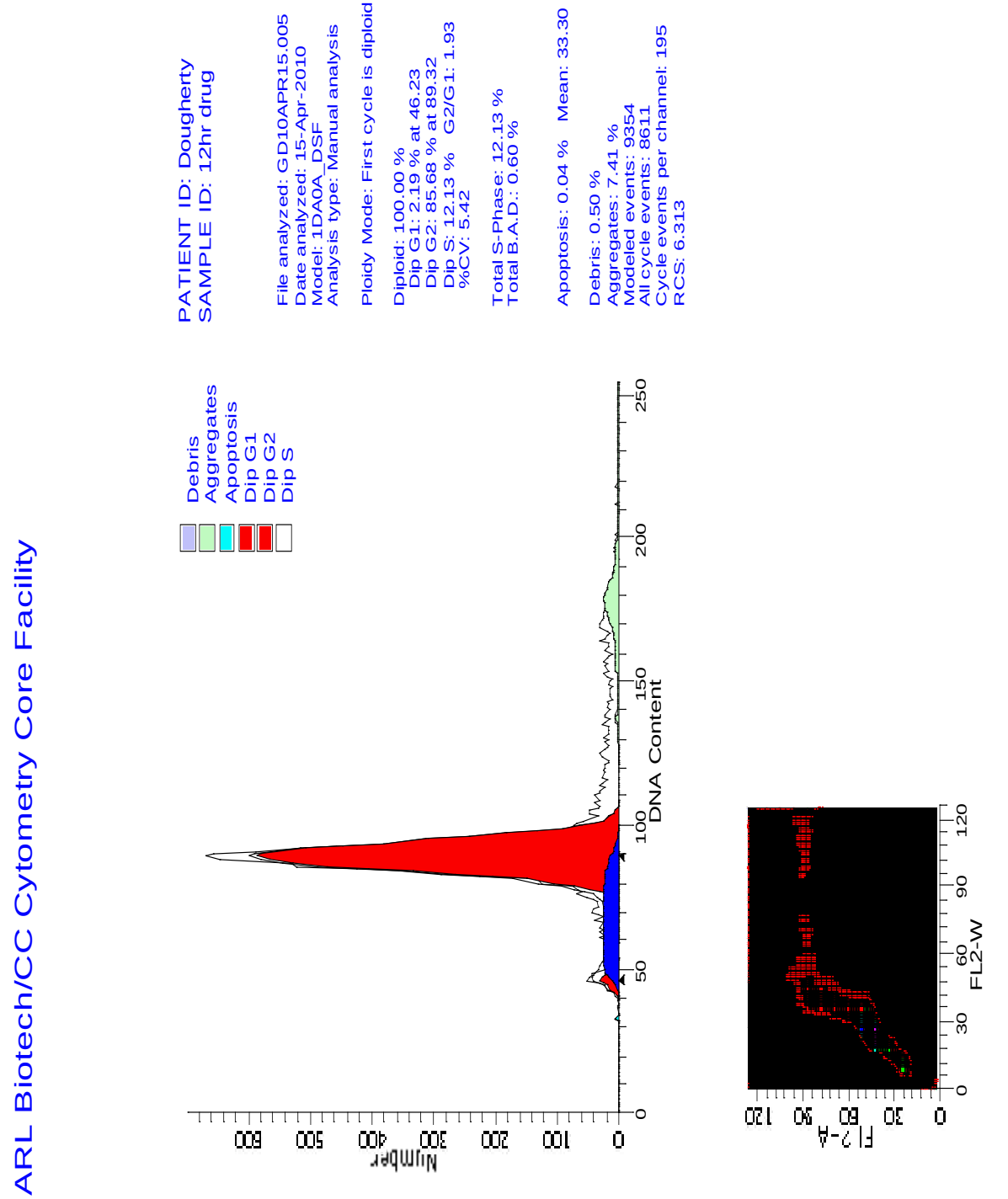

ARL Biotech/CC Cytometry Core Facility
PATIENT ID: Dougherty
SAMPLE ID: 12hr drug
File analyzed: GD10APR15.005
Date analyzed: 15-Apr-2010
Model: 1DA0A_DSF
Analysis type: Manual analysis
Ploidy Mode: First cycle is diploid
Diploid: 100.00 %
Dip G1: 2.19 % at 46.23
Dip G2: 85.68 % at 89.32
Dip S: 12.13 % G2/G1: 1.93
%CV: 5.42
Total S-Phase: 12.13 %
Total B.A.D.: 0.60 %
Apoptosis: 0.04 % Mean: 33.30
Debris: 0.50 %
Aggregates: 7.41 %
Modeled events: 9354
All cycle events: 8611
Cycle events per channel: 195
RCS: 6.313
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
Number
DNA Content
FL2-A
FL2-W
ModFitLT V3.1(PMac)

Figure 8

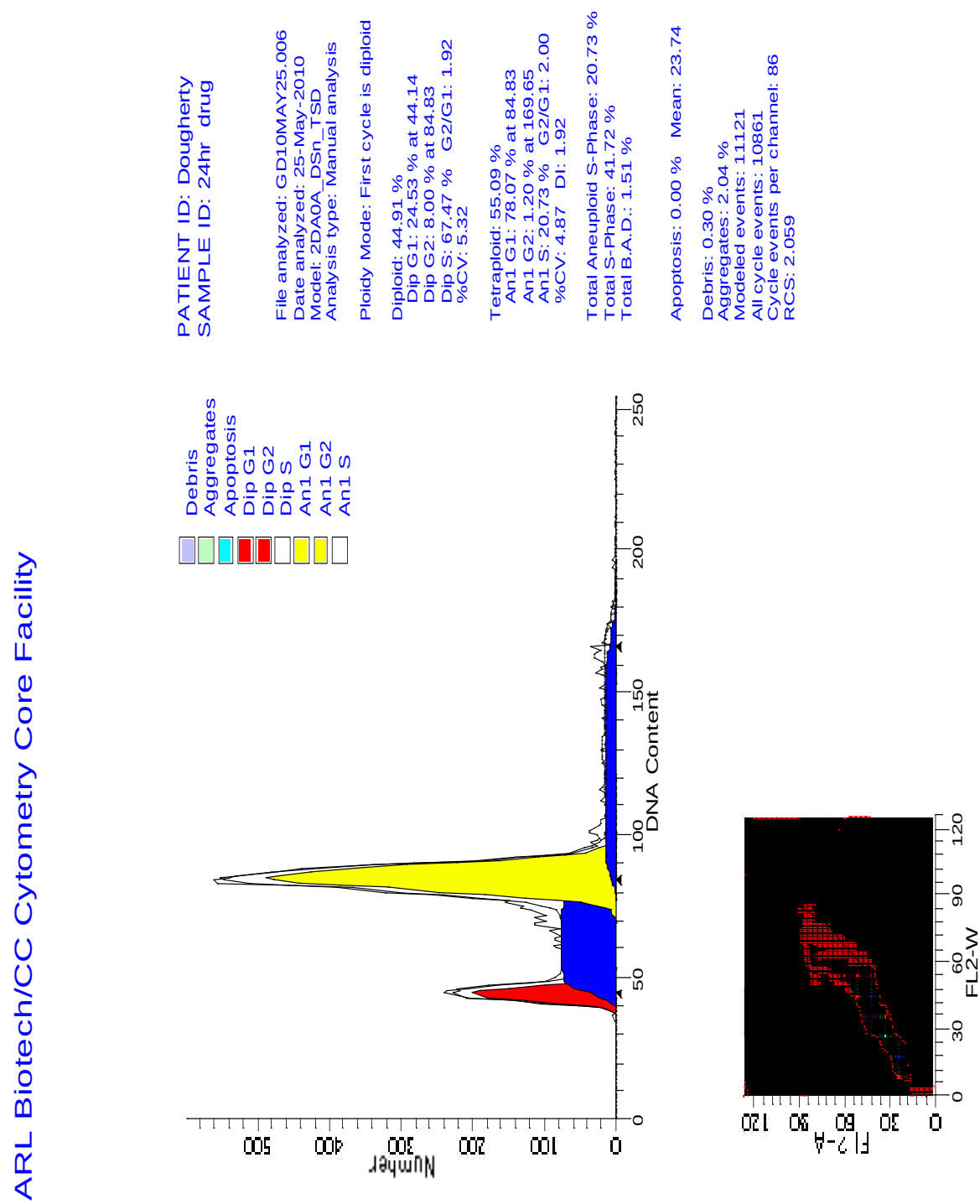

ARL Biotech/CC Cytometry Core Facility
PATIENT ID: Dougherty
SAMPLE ID: 24hr drug
File analyzed: GD10MAY25.006
Date analyzed: 25-May-2010
Model: 2DA0A_DSn_TSD
Analysis type: Manual analysis
Ploidy Mode: First cycle is diploid
Diploid: 44.91 %
Dip G1: 24.53 % at 44.14
Dip G2: 8.00 % at 84.83
Dip S: 67.47 % G2/G1: 1.92
%CV: 5.32
Tetraploid: 55.09 %
An1 G1: 78.07 % at 84.83
An1 G2: 1.20 % at 169.65
An1 S: 20.73 % G2/G1: 2.00
%CV: 4.87 DI: 1.92
Total Aneuploid S-Phase: 20.73 %
Total S-Phase: 41.72 %
Total B.A.D.: 1.51 %
Apoptosis: 0.00 % Mean: 23.74
Debris: 0.30 %
Aggregates: 2.04 %
Modeled events: 11121
All cycle events: 10861
Cycle events per channel: 86
RCS: 2.059
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
An1 G1
An1 G2
An1 S
Number
DNA Content
FL2-A
FL2-W
ModFitLT V3.1(PMac)

Figure 9

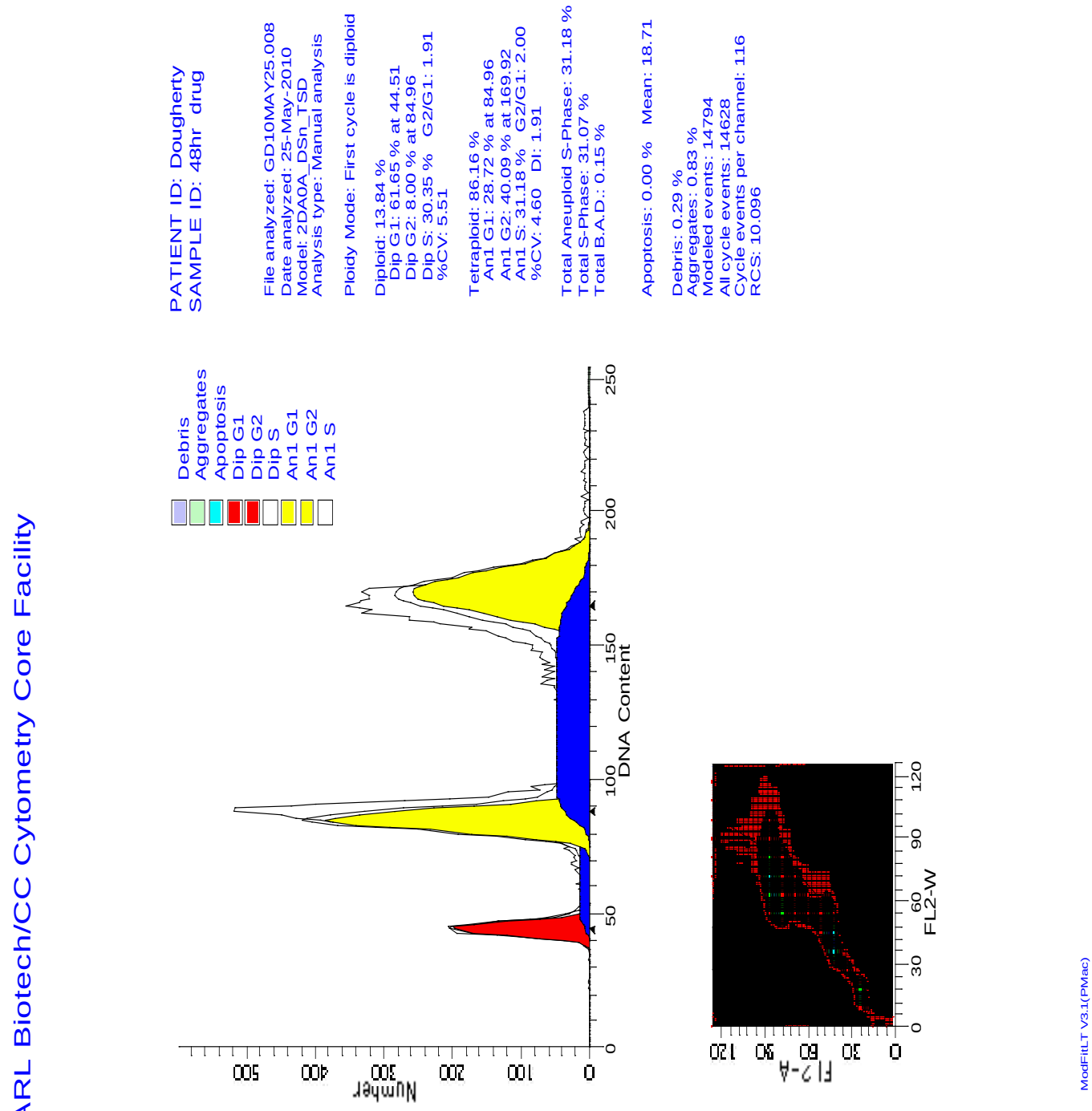
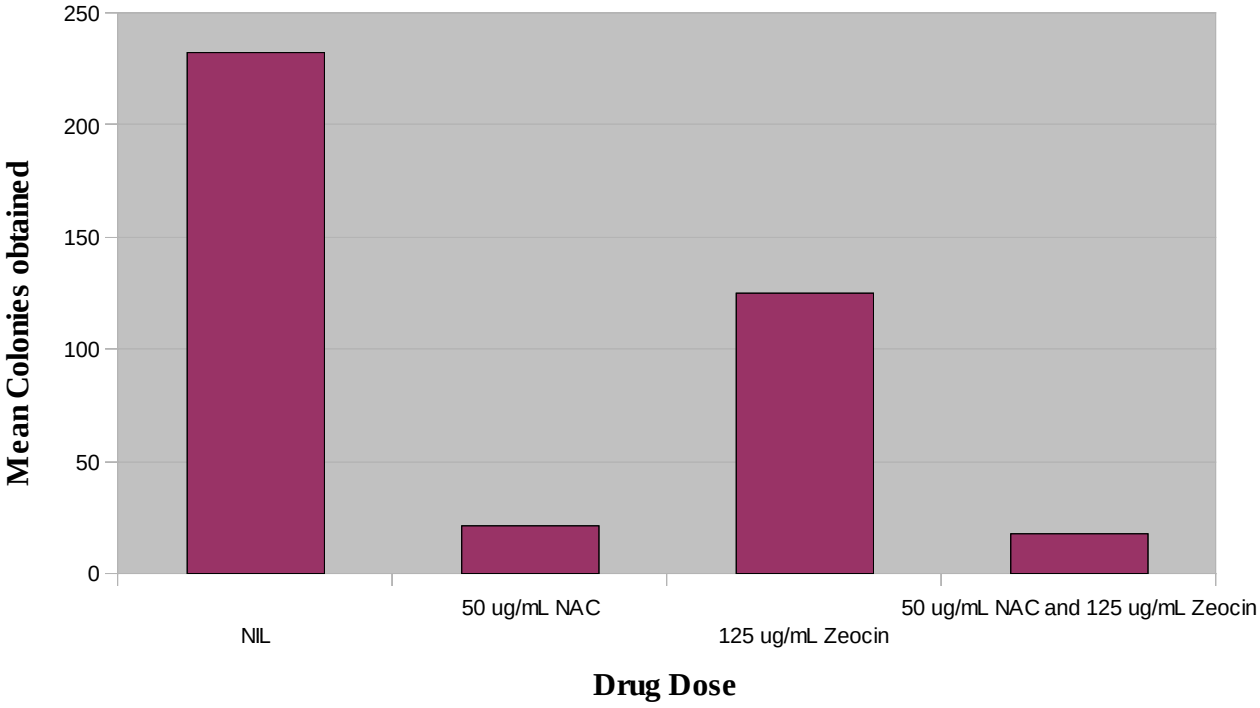

ARL Biotech/CC Cytometry Core Facility
PATIENT ID: Dougherty
SAMPLE ID: 48hr drug
File analyzed: GD10MAY25.008
Date analyzed: 25-May-2010
Model: 2DA0A_DSn_TSD
Analysis type: Manual analysis
Ploidy Mode: First cycle is diploid
Diploid: 13.84 %
Dip G1: 61.65 % at 44.51
Dip G2: 8.00 % at 84.96
Dip S: 30.35 % G2/G1: 1.91
%CV: 5.51
Tetraploid: 86.16 %
An1 G1: 28.72 % at 84.96
An1 G2: 40.09 % at 169.92
An1 S: 31.18 % G2/G1: 2.00
%CV: 4.60 DI: 1.91
Total Aneuploid S-Phase: 31.18 %
Total S-Phase: 31.07 %
Total B.A.D.: 0.15 %
Apoptosis: 0.00 % Mean: 18.71
Debris: 0.29 %
Aggregates: 0.83 %
Modeled events: 14794
All cycle events: 14628
Cycle events per channel: 116
RCS: 10.096
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
An1 G1
An1 G2
An1 S
Number
DNA Content
FL2-A
FL2-W
ModFitLT V3.1(PMac)

Figure 10


250
200
150
100
50
0
Mean Colonies obtained
NIL
50 ug/mL NAC
125 ug/mL Zeocin
50 ug/mL NAC and 125 ug/mL Zeocin
Drug Dose

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