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Determining protein biomarkers for chemotherapeutic drug resistance in breast cancer

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DETERMINING PROTEIN BIOMARKERS FOR CHEMOTHERAPEUTIC DRUG RESISTANCE IN BREAST CANCER

by

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ABSTRACT

Determining Protein Biomarkers for Chemotherapeutic Drug Resistance in Breast Cancer

by

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Although there have been many research advances in the treatment and prevention of cancer, it remains a leading cause of death each year throughout the world. A major obstacle in the treatment of cancer is encountered when the cancer develops resistance to chemotherapeutic agents. To better understand resistance and identify biomarkers we propose to develop resistance in breast adenocarcinoma cancer cell lines, MDA-MB-231 and MCF-7, using common chemotherapeutic agents, doxorubicin and cisplatin. Microarray analysis will be preformed to identify over expression of proteins in these resistant lines. We hypothesize that genes which are over-expressed in a resistant cancer cell line may be responsible for the observed chemoresistance. To test this hypothesis, chemical inhibitors will be used to suppress protein expression in resistant and wild type cell lines, and then perform proliferation and viability assays to observe changes in protein synthesis from over expressed genes.

Our preliminary results indicated 10 genes which were highly over-expressed in the resistant cancer cell line SD231RD2 in comparison to the MDA-MB-231 wild type

cell line. We chose to focus on two genes, reported in the literature to be commonly expressed in response to stress, resulting in increased production of cyclooxygenase-2 (COX-2) and metalloproteinase-3 (MMP-3). Inhibition of COX-2 protein with indomethacin using clonogenic survivals reversed chemoresistance in resistant cells.

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ABBREVIATIONS

BCL2	B-cell CLL/lymphoma 2
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BSA	Bovine Serum Albumin
CDDP	Cis-diamine dichloroplatinum
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOX	Doxorubicin
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
EtBr	Ethylene Bromide
FBS	Fetal Bovine Serum
GDAS	GeneChip DNA analysis software
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic Acid
HRP	Horseradish Peroxidase
IL-11	Interleukin -11
IL-1B	Interleukin -1β
IL-8	Interleukin -8
M-PER	Mammalian Protein Extraction Reagent
mRNA	messenger Ribonucleic Acid
OH	Hydroxyl groups
PAGE	Polyacrylamide gel Electrophoresis
PBS	Phosphate Buffered Saline
PGE_2	Prostaglandin E2
P-gp	P-glycoprotein
PI3-K/Akt	Phosphatidylinositol-3-kinase
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate

SEM	Standard Error of the Mean
SQ-PCR	Semi-Quantitative Polymerase Chain Reaction
TBST	Tris Buffered Saline with Tween20
TIMP	Tissue Inhibitor metalloproteinase

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I want to thank my family and my friends for always being there for me by providing support whenever desired. Additionally, I would like to thank the professors who stepped in and helped our research group when it seemed like we wouldn't make it to this point.

Mostly, I would like to dedicate this thesis to the loving memory of Dr. Stephen Carper. I want to thank him for allowing me to work under his supervision, showing me my strengths and guiding me throughout my masters degree, always encouraging me to strive to reach my goals, and showing me the utmost confidence in my research capabilities. I am sincerely grateful for everything he has instilled in me and hope that one day I can be half the scientist he was. There are no words to describe the loss of a professor, but I feel blessed for the time that I had with him and will forever be grateful for all the knowledge that he has given me in research and in life. I hope you are proud of everything that your students accomplish and you are sorely missed.

CHAPTER 1

INTRODUCTION

1.1 Purpose of the Study

The development of the first documented anticancer agent occurred in 1943 when Dr. Louis Goodman and Dr. Alfred Gillman reported that the alkylating agent nitrogen mustard could induce regression of lymph node enlargement in malignant lymphoma (1). Our ongoing battle to discover new efficient treatments for cancer continues today, with cancer being responsible for one in every four deaths in America. The American Cancer Society estimates that there will be over 1.4 million new cases of cancer diagnosed in 2008 and half of those are expected to die from the disease. Approximately 185,000 of these estimated cases will be diagnosed with breast cancer (2).

Breast cancer is positively correlated with age, an onset of menses that occurs early in life and ends late in life, never having children or having the first child after the age of thirty, and inherited genetic mutations in *BRCA1* and *BRCA2*. Treatments for breast cancer include surgery (partial or full mastectomy), chemotherapy, radiation therapy and hormone therapy (2). Although treatment options are available to patients that have been diagnosed with cancer, in recent years there have been no advances in gene therapy for patients. Because anticancer agents have different efficacies among patients due to individual differences in gene expression profiles, predicting productive outcomes in

patients remains problematic (3). Another major drawback of using chemotherapy is that cancers may develop resistance to therapy due to changes in gene expression. Also, patients undergoing treatment with anticancer agents may be initially resistant to a specific treatment or may become resistant. If this occurs, the patient's cancer may progress which requires utilization of alternate therapies. If multi drug resistance occurs the typical outcome for the patient is death.

1.2 Significance of Study

The development of an *in vitro* resistant cancer cell line to a specific chemotherapeutic agent can provide detailed gene expression patterns that will demonstrate potential protein biomarkers that may be responsible for cancer resistance. This experimental outcome could potentially improve selection of effective therapy strategies for individual cancer patients. Successful therapy would follow after examination of gene expression profiles of tumors to specify which genes are over or under expressed. The physician would then treat the patient with the anticancer agent that would best combat that type of cancer based on factors such as the gene expressed that would render a patient sensitive or resistant to specific anticancer agents. The results from a gene expression profile could also allow use of blocking agents to inhibit genes that are responsible for chemoresistance.

1.3 Research Tasks

To determine gene expression profiles and possible biomarkers for treatment of chemoresistant breast cancers, data will be collected from the following tasks performed:

1. Preparation of a resistant breast cancer cell line;
2. Determine by microarray analysis which genes are overexpressed in the resistant cancer cell line compared to wild type;
3. Identify the pathway or mechanism involving the overexpressed genes;
4. Determine if an inhibitor of protein expression will restore sensitivity to the anticancer agent in the resistant line.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 Gene Expression in Resistant Cancer

Microarray analysis has been performed on multiple cancer cell lines and tumors treated with varying anticancer agents *in vitro* to determine which genes are responsible for resistance or sensitivity to specific chemotherapeutic agents. The results allow researchers to determine if drugs with similar modes of action will regulate the same clusters of genes involved in chemoresistance (4). Cancer chemotherapy is limited by the emergence of chemotherapeutic resistant tumor cells. For example, the gene identified as MDR is a multidrug resistant gene that exhibits cross resistance to a variety of anticancer agents when P-glycoprotein (P-gp) is over expressed. This protein is responsible for increasing the removal of the anticancer agent from the cell, thereby reducing the efficacy of the drug (5). Researchers have found a variety of genes specific for each cancer type that may cause resistance to specific anticancer agents, *in-vitro*. Once resistance develops in a cell line the possibility of multidrug resistance also increases, which limits treatment success.

2.2 Wild Type vs. Resistant Cancer Cell lines

Two human breast adenocarcinoma cell lines, MDA-MB-231 and MCF-7, are the main targets of interest and are used as model systems. MDA-MB-231 was established from a biopsy of a tumor from the breast of a 51 year old Caucasian female, and MCF-7 was established from a biopsy of a tumor from the breast of a 69 year old Caucasian female (www.atcc.org). These cell lines differ in one main aspect: the MCF-7 cancer cell line is positive for an estrogen receptor (ER^+), whereas the MDA-MB-231does not express an estrogen receptor (ER^-). These cell lines differ in their responses to chemotherapy, which allows resistance to develop in one cell line but not in another due to differing gene expressions (6). It is also predicted that tumors which are ER^- have a poorer prognosis following chemotherapy compared to patients with ER^+ tumors who have longer, disease free survival rates (7).

Our goal is to develop MDA-MB-231 and MCF-7 cell lines resistant to anticancer agents without manipulating the presence of specific genes. This approach differs from the typical method in which cells lines have specific genes removed, resulting in resistance to specific anticancer agents. For example, AdrR MCF-7 is resistant to adriamycin up to10 μM, which produces resistance to benzo(a)pyrene (8). Instead of purchasing a cell line that is resistant to a specific anticancer agent, we chose to convert the wild type cell lines MDA-MB-231 and MCF-7 to resistant lines by continual exposure to either doxorubicin or cisplatin. By producing these cell lines in this manner, we can determine which genes are responsible for the development of resistance to anticancer agents. This approach allows systematic adaptation for resistance within the biological system, rather than artificial manipulation achieved by intentional genetic

change, to determine the factors which are responsible for resistance to chemotherapeutics. The process of selecting resistant clones is described in Figure 1.

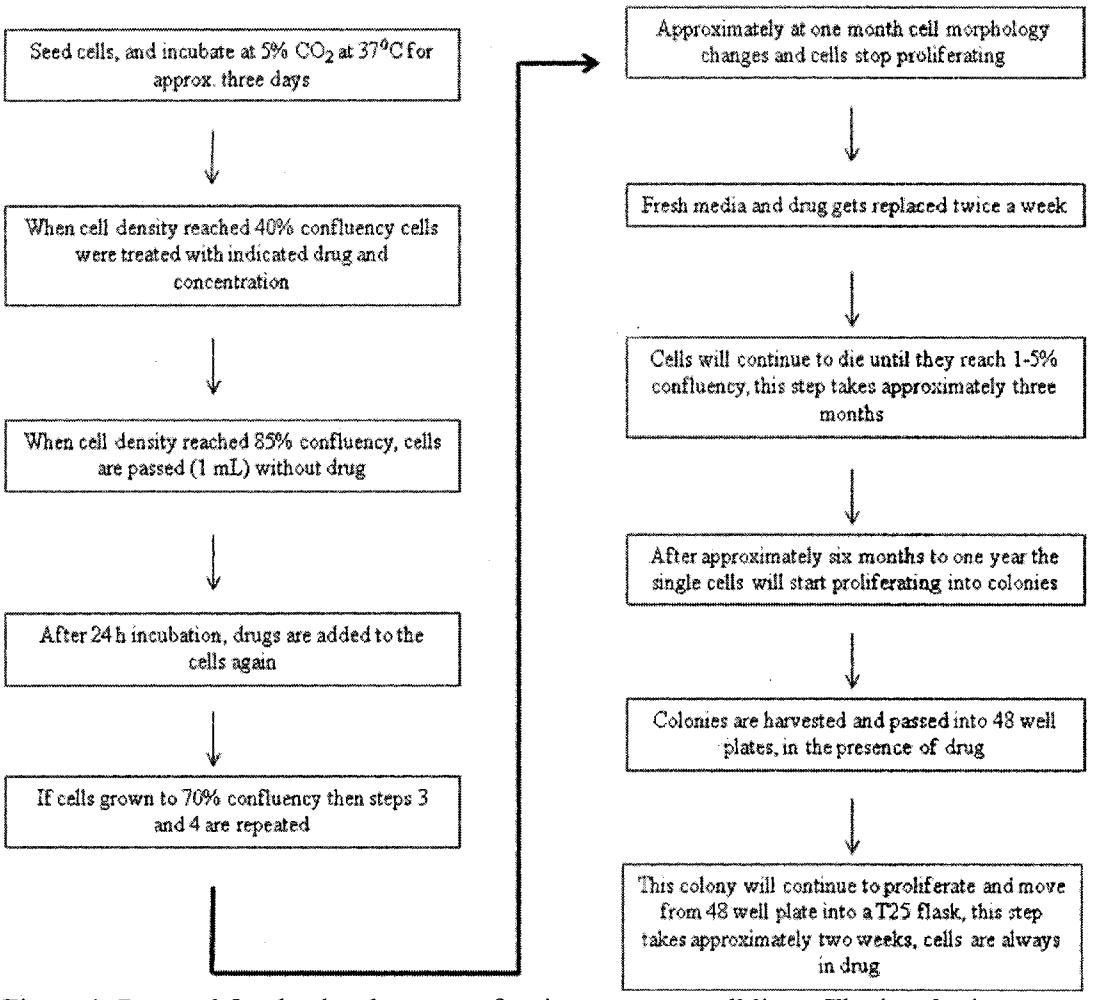


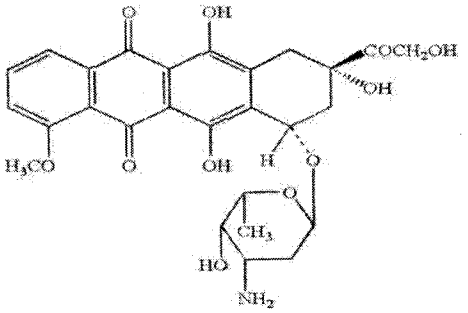
Figure 1. Protocol for the development of resistant cancer cell lines. The incubation environment is maintained at 37°C, 95% relative humidity and 5% CO_2.

2.3 Cisplatin and Doxorubicin

Cisplatin and doxorubicin are common anticancer agents used in chemotherapy regimens. However, cancer can become resistant to these anticancer agents. The

structures of these compounds are illustrated in Figure 2. Overcoming resistance by finding novel biomarkers will aid with early detection to avoid development of resistant tumors or allow for quicker detection of resistance providing an earlier opportunity to alter the chemotherapy strategy. To better understand the mechanism of action requires genetic profiling within resistant tumors to determine the cause of resistance.

A



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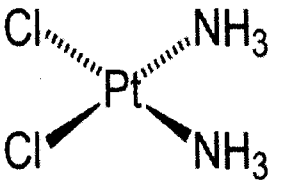

Figure 2. Structures of chemotherapeutic agents. Doxorubicin (DOX) chemical structure is shown in A. (Source:http://www.freepatentsonline.com/6653455-0-display.jpg), while cisplatin (CDDP) chemical structure is shown in B.
(Source:http://upload.wikimedia.org/).

The lethality of cisplatin is attributable to irreversible adduct formation to the DNA backbone through a reaction with the seventh nitrogen (N7) of adjacent guanines (Figure 3). Cisplatin can enter the cell by passive diffusion or active uptake. Passive diffusion can occur because the chloride ion concentration is much lower inside the cell then on the outside. Once in the cell, cisplatin undergoes hydrolysis and exchanges its chloride atoms for hydroxyl (OH) groups. The OH groups will then be able to make interstrand and intrastrand crosslinks to the seventh nitrogen (N7) of adjacent guanines (Figure 3) to form a bulky lesion in the DNA backbone which ultimately results in the recruitment of DNA repair mechanisms. If the lesion cannot be repaired, the cell

undergoes programmed cell death via the apoptotic pathway (9-10). Some mechanisms involved in the cellular resistance of cisplatin include reduced drug uptake, increased drug efflux, and increased DNA repair (11). For example, in the case of reduced drug uptake 90% of cisplatin when given intravenously will bind to plasma proteins, thus the agent never reaches target cells, leading to inactivation of many cisplatin molecules (10). If products of gene expressions in these pathways could be indentified it is possible the genes could be targets for monitoring resistance.

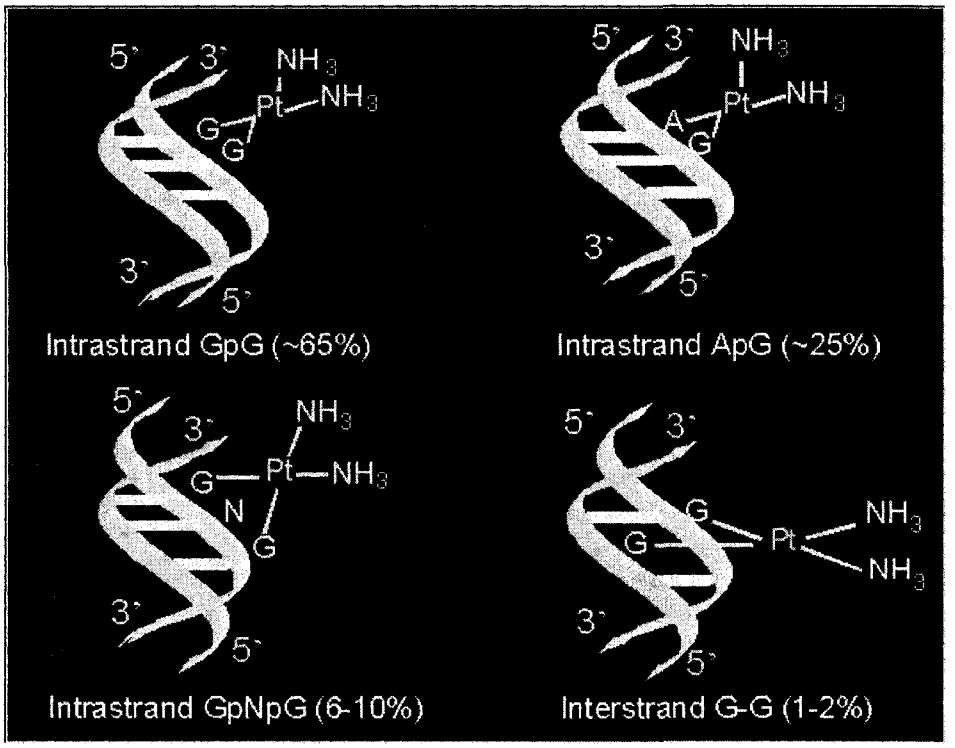

Figure 3. Cisplatin interaction to the N7 of guanine or adenine on DNA backbone. Percentages represent how many adducts are formed with the specific crosslink stated. (Source:www.jcu.edu.au/.../research/cisplatinweb.htm)

The effects of doxorubicin are complex, but one nuclear action is mediated by indirect inhibition of topoisomerase II activity (Figure 4). The drug interferes with DNA winding by intercalation between the double helix which interferes with the ability of topoisomerase II to unwind DNA for successful transcription (Figure 5). It has been found that a decreased level of topoisomerase II hinders the efficacy of doxorubicin (12), since there is lowered target that is available for doxorubicin. Additional data suggests that doxorubicin has the ability to induce DNA strand breaks which causes cell death through free radical mediated apoptosis (13).

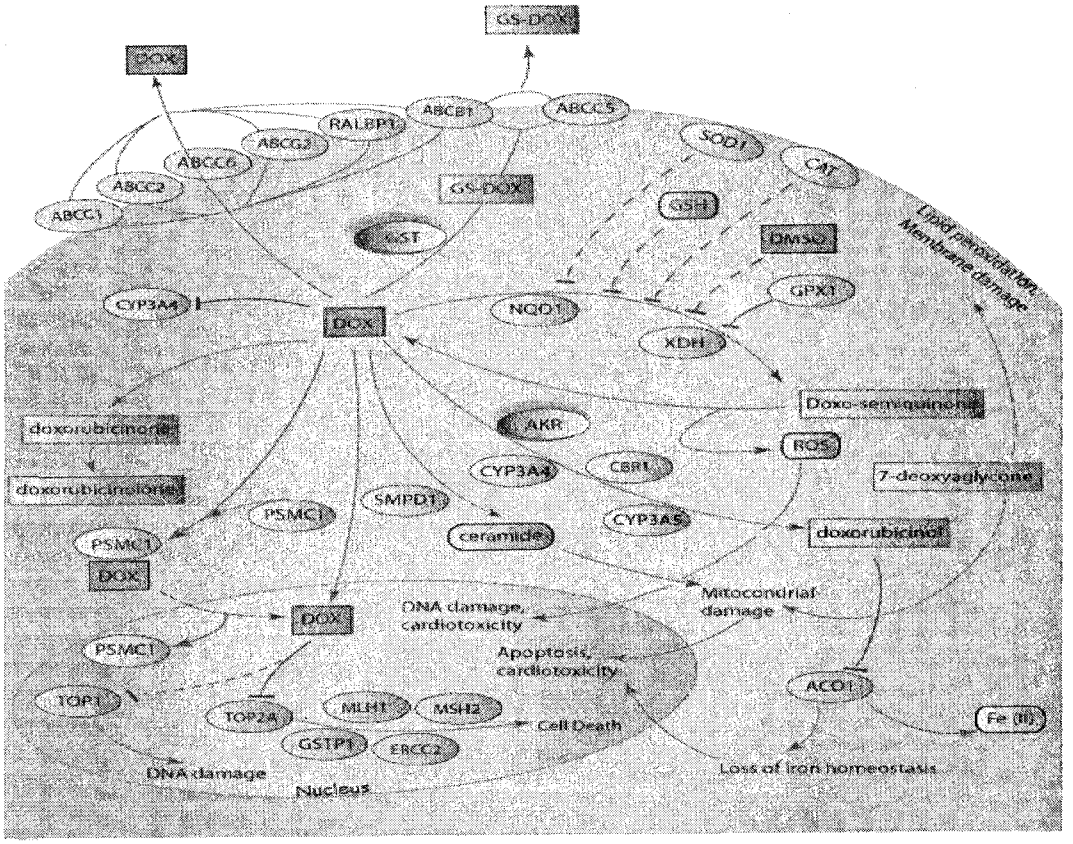

Figure 4. Mechanism and pathways affected when doxorubicin enters the cancerous cell. (Source:http://www.pharmgkb.org/do/serve?objId=PA155618789&objCls=Pathway)

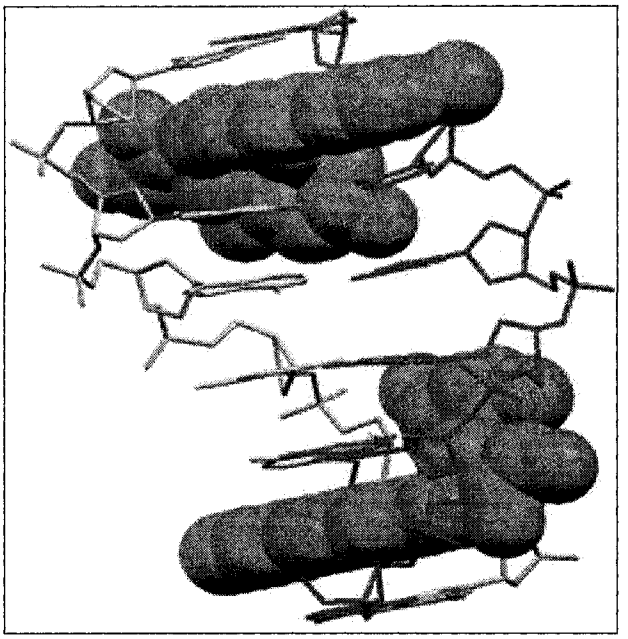

Figure 5. Doxorubicin intercalation with DNA backbone. It is proposed that intercalation with doxorubicin will not allow for transcription therefore signalling programmed death in tumor cells. (Source:http://www.rsc.org/ej/CS/2007/b606046n/b606046n-f13.gif)

2.4 Genes of Interest

From the microarray data two proteins of interest were discovered. The first gene of interest is cyclooxygenase-2 (COX-2 also known as PGH synthase), a glycoprotein regulated at both transcription and post transcriptional levels by proinflammatory agents, cytokines, growth factors, oncogenes and tumor promoters. It is known that COX-2 is expressed in the early stages of carcinogenesis (14). As shown in Figure 6, COX-2 is involved in the conversion of arachidonic acid into prostaglandins. This conversion is crucial since prostaglandin E_2 (PGE_2), one of the end products of the arachidonic acid pathway, is also responsible for mitogenesis and tumor growth (15). COX-2 expression activates the phosphatidyl inositol 3-kinase/AKT signalling pathway, a biochemical pathway which signals for cell survival (16). COX-2 and PGE_2 play important roles in

regulating tumor invasion, angiogenesis and apoptotic resistance. In addition, COX-2 and PGE_2 each promote malignant growth (17).

The other gene of interest is matrix metalloproteinase-3 (MMP-3 or stromelysin). This protein is synthesized and secreted from connective tissue cells and degrades several extracellular matrix molecules (18). MMP-3 is known to promote tumor formation and cell proliferation by release of growth factors, and has been shown to impede tumor relevant processes such as apoptosis (19).

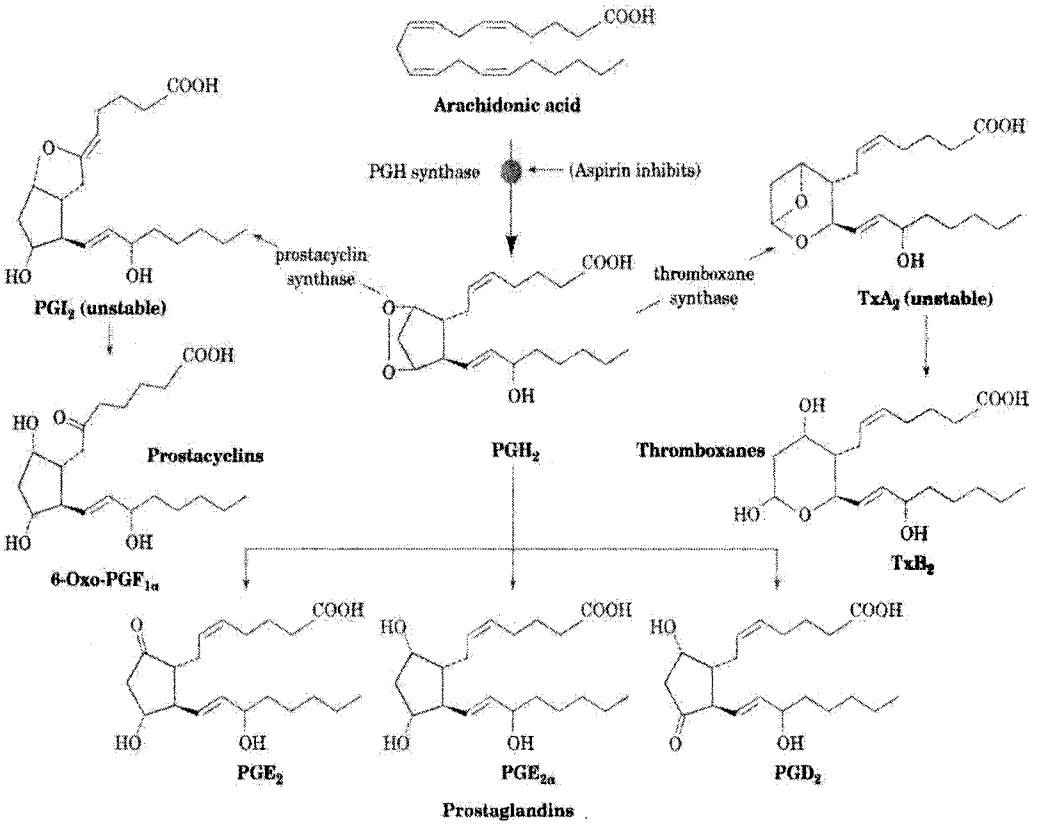

Figure 6. The cyclic pathway of arachidonic acid metabolism (20). The pathway branches demonstrate synthesis of the prostaglandin of interest PGE_2. PGH synthase is also known as COX-2.

2.5 Hypothesis

Although advances are being made in treatments provided for patients with cancer, many patients still have a poor prognosis due to chemoresistance. It is important to try to determine what causes resistance in human cancer cell lines and tumors. If we could determine which proteins are responsible for resistance in cancer cells we could provide a better treatment option for the patient. Our hypothesis is that a small subset of proteins provide protection against a wide variety of anticancer agents, knowing these proteins will allow us to determine better chemotherapeutic treatments for patients. To test this hypothesis two established breast cancer cell lines MCF-7 and MDA-MB-231 will be made resistant to two common chemotherapeutic agents cisplatin and doxorubicin. The resistance to these agents will be confirmed by proliferation and viability assays. Once established, these resistant cell lines will be analyzed by microarray analysis to reveal over and under expressed gene expressions. Gene expression of the resistant cells will be compared to parental cell lines. Those gene messages showing the largest increase in expression will be investigated further. The importance of elevated messages will be investigated by inhibiting the function (chemical inhibitor) of the protein.

CHAPTER 3

MATERIALS AND METHODS

The samples and drug preparations that required the use of water were completed using ultrapure water. All chemicals and reagents are of highest quality grade. All cancer cell lines were maintained at 95% humidity, 5% CO_2 and 37 ^{o}C unless otherwise stated.

3.1 Cell Culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 and MCF-7 were both cultured in MEM medium (containing Earle's Salts and L-glutamine, Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), 25mM HEPES pH 7.4 (Sigma-Aldrich, USA) and 1% penicillin-streptomycin (Invitrogen, USA). Resistant lines were also cultured in MEM medium with either 0.01 μM doxorubicin (Sigma-Aldrich, USA) or cisplatin (Sigma-Aldrich, USA). Cells were incubated at 5% CO_2 and 37 ^{o}C in 95% humidity. Cell harvests were obtained by washing cells three times in phosphate buffered saline (PBS at pH7.2) which did not contain $CaCl_2$ or $MgCl_2$ (Invitrogen, USA) and chemically detaching adherent cells from the surface of the flask using trypsin-EDTA (0.25% Trypsin with 53mM EDTA) in HBSS without calcium and magnesium (ATCC, Manassas, VA) for ten min. Fresh medium was added

to inactivate trypsin. Cells counts were performed using a Z1 Beckman Coulter® Particle Counter (Fullerton, CA).

3.2 Z1 Beckman Coulter® Particle Counter

To determine cell numbers in cell suspensions (0.1 mL) of attached cells was added to a coulter counter cup containing 9.9 mL of isotonic diluent azide-free (Isoton) solution (Val-Tech Diagnostics Inc., Pittsburgh, PA). The solution was stirred and placed in the Z1 Beckman Coulter® Particle Counter (Fullerton, CA). Two or three measurements were averaged and used to calculate the number of cells per mL of solution.

3.3 Preparation of Cisplatin, Doxorubicin and Indomethacin

A 50 mM stock of cisplatin was prepared by dissolving 0.150 grams of 300.05 g/mol cisplatin (CDDP; Sigma-Aldrich, USA) in 10 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). The solution was filtered using a syringe (Becton Dickinson, USA) under sterile conditions with a 0.2 μM nylon membrane syringe filter (PALL, Ann Arbor, MI). Serial dilutions were made using 500 μL of the 50 mM stock diluted in 4.5 mL of sterile filtered DMSO.

A 5 mM stock of doxorubicin was prepared by dissolving 0.029 grams of 579.98 g/mol doxorubicin (DOX; Sigma-Aldrich, USA) in 10 mL of water. The solution was filtered using a syringe under sterile conditions with a 0.2 μM polyethersulfone membrane syringe filter (VWR International, USA). Serial dilutions were made using 500 μL of the 50 mM stock diluted in 4.5 mL of sterile filtered water.

A 500 mM stock of indomethacin was prepared by dissolving 1.789 grams of 357.79 g/mol indomethacin (Sigma-Aldrich, USA) in 10 mL of DMSO. The solution was filtered using the aforementioned procedure. Serial dilutions were made using 500 µL of the 500 mM stock diluted in 4.5 mL of sterile filtered DMSO.

3.4 Mitochondrial Dehydrogenase Assay

Cellular mitochondrial dehydrogenase activity was determined using the AlamarBlue assay (BioSource, Camarillo, CA). Varying amounts of cells were seeded in a 96-well plate in 0.1 mL aliquots and allowed to adhere for an incubation time of 24 h. Triplicate wells were exposed to varying concentrations of either doxorubicin or cisplatin prepared in cell culture medium. The first row of the plate did not contain cells and was used as a blank measurement. Following exposure to test substances, plates were incubated for 24 and 48 hour time periods. After incubation, 0.1 mL fresh cell culture media, and 0.01 mL AlamarBlue was added to each well. The plates were then incubated for 4 h. Absorbance of each well was measured using a GENios fluorescence plate reader (Tecan Systems Inc. San Jose, CA) at excitation and emission wavelengths of 530 nm and 590 nm, respectively. Percent viability was reported as a percent of controls (n=3).

3.5 Colony Formation Assay (Clonogenic Survival)

Tissue culture flasks (25 cm^2) were seeded with 50 x 10^3 (MDA-MB-231) cells, 150 x 10^3 (SD231RD2) cells or 150 x 10^3 (MCF-7) cells and incubated for 72 h. After this incubation period, the cells were treated with varying concentrations of doxorubicin or cisplatin and incubated for 1 h or 24 h (control cells were treated with the drug vehicle).

Cells were harvested, counted, and dilutions were prepared such that 150 cells (MDA-MB-231), 300 cells (SD231RD2) or 200 cells (MCF-7) were sub-cultured in triplicate in 60 mm x 15 mm cell culture dishes (Corning Inc., USA). Dishes were incubated for 10 days, and stained with crystal violet (0.5% weight/volume crystal violet in 95% ethanol, Sigma-Aldrich, USA), and colonies (n > 50 cells) counted. Percent survival compared to control cells was measured (n = 3).

Indomethacin inhibition of COX-2 survival: To maintain consistent confluencies, tissue culture flasks (25 cm^2) were seeded with 50 x 10^3 (MDA-MB-231) cells and incubated for 72 h, while flaks containing SD231RD2 were seeded with 150 x 10^3 cells and incubated for 48 h. After this incubation period cells were treated with varying concentrations of doxorubicin and 50 μM indomethacin then incubated for 24 h (control cells were treated with drug vehicle). Other controls included triplicate plates treated only with the lethal dose 50% (LD_{50}) of doxorubicin, and triplicate plates treated only with indomethacin. Cells were harvested, counted, and dilutions were prepared such that 150 cells (MDA-MB-231), 300 cells (SD231RD2) were plated in triplicate in 60 mm x 15 mm cell culture dishes. Dishes were incubated for 10 days, stained with crystal violet, and colonies (n > 50 cells) counted. Percent survival compared to control cells was measured (n = 3).

3.6 Six Day Viability Assay

Tissue culture flasks (25 cm^2) were seeded in triplicate for each drug time with 50 x 10^3 (MDA-MB-231) cells and 150 x 10^3 (SD231RD2). Cells were maintained in MEM and incubated for 24 h (day 0). After this incubation period cells were treated, referred as

day one, with varying concentrations of doxorubicin (control cells received water). Flasks were then incubated for two more days. On day three, each of the flasks were harvested separately and re-seeded in one-eighth of the volume (625 μL) into a new flask containing 4275 μL of MEM and 100 μL of doxorubicin drug (specific concentration for each drug point). Flasks were then incubated for three more days. On day six, cells from each flask were harvested separately and the cell density was determined in cells/mL by conducting cell counts.

3.7 RNA Extractions

Cells were passed at the same time each day for a total of three passages and maintained at 50-60% confluency. Media was removed from the triplicate flasks containing MDA-MB-231 or SD231RD2 and rinsed three times with 5 mL of PBS. Cells were then harvested and transferred to a 15 mL conical vial. Total RNA extractions were then performed according to protocol using QIAGEN RNeasy MINI Kit purification system (QIAGEN, USA). The resulting solution was separated into aliquots for the purpose of microarray analysis (Windber), Polymerase Chain Reactions (PCR), quantitation, and verification of purity. The aliquots are immediately stored at -70°C until further use.

3.8 Instructions for RNA Quantification and Purification on NanoDrop

To ensure the integrity of the RNA, all samples remained on ice and were handled using RNase free materials. A 1:9 dilution of sample to Tris buffer was prepared for the purpose of verifying sample purity The NanoDrop (ND-1000 spectrophotometer, Thermo

Scientific, USA) instrument was set to zero using RNase free water prior to measuring the absorbance of the samples. A total of 2 μL of sample was loaded onto the sensor to measure the absorbance at 260 and 280 nm. The sensor was thoroughly cleaned using RNase free water between sample readings. Each sample was measured twice and the average measurement is used in data analysis. Concentration of RNA was calculated by the NanoDrop software. To verify purity, the samples diluted in Tris buffer was measured and only samples with an A260/280 reading within 1.8-2.3 were used for microarray analysis.

3.9 Microarray Analysis

Microarray Analysis was performed by Windber Research Institute (Windber, PA) using Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) for this study. It is a single array with over 47,000 transcript probe sets representing over 38,500 well-substantiated human genes. Windber followed the Affymetrix GeneChip® Expression Analysis Technical Manual for all GeneChip array procedures. Briefly, one μg of total RNA was used for reverse transcription to produce single strand cDNA followed by second strand synthesis to form double strand cDNA. After cDNA purification, biotin-labeled aRNA target wass produced by an *in vitro* transcription (IVT) reaction using the cDNA template. After aRNA purification, an aliquot of the labeled aRNA is run on Agilent's Bioanalyzer as a quality check and another aliquot is quantified using the NanoDrop UV/Vis spectrophotometer (NanoDrop). Only high quality RNA with a yield of more than 10 μg was fragmented and hybridized to Affymetrix GeneChip arrays overnight (18 hours) in a temperature-controlled hyb-

oven. After hybridization, GeneChip arrays were loaded onto a Fluidic Station 450 for washing and staining using the standard Affymetrix procedure. After the final wash, the GeneChip arrays were scanned using the Affymetrix GeneChip scanner 3000 G7. Scanned images were analyzed using Affymetrix data analysis software (GDAS) to generate the raw data.

Data analysis included the use of GDAS to generate many different files for each array. Windber imported the CEL file into GeneSpring microarray analysis software (Agilent). All six arrays were subjected to GCRMA processing then through log transformation and global normalization to generate normalized data. Probes were eliminated with raw signal intensity below 50 in four out of six samples, resulting in 20,500 probe sets for further analysis. Because there were not enough replicates for each treatment, fold change was used (two fold as cut off) to identify differentially expressed genes for each comparison. The probe sets that were at least two fold different were further reduced by eliminating inconsistent points; i.e., if normalized signal value in one of the replicates is determined to be similar to the other group, it is considered a potential false positive. For instance, (A1: 50; A2: 56) compare to (B1: 50; B2: 06). Average of A and B are 53 and 23, respectively. It is two-fold percent different between A and B (53/23 = 2.3). However, this specific data point is not included because the value of B1 is 50. The t-test was used to further reduce false positive probe sets when multiple replicates were available.

3.10 Western Blot Analysis

MDA-MB-231 (250×10^3) cells or SD231RD2 (400×10^3) cells were seeded into a 75 cm^2 flask and maintained with a total volume of 10 mL of MEM cell culture medium. The resistant line (SD231RD2) was also supplemented with 200 μL of 0.0005 mM stock of doxorubicin, for a final concentration of 0.01 μM doxorubicin. After 3 days of incubation, the adherent cells were washed three times with 10 mL of PBS, discarding each wash. Following the washing procedure, 5 mL of PBS was added to the flask and cells were scraped and placed into a 15 mL conical tube. The cells were centrifuged at room temperature for seven minutes at 1,500 rpm and supernatant discarded. To the remaining cell pellet, 500 μL of M-PER® cell lysis buffer (Pierce Rockford, IL) was added in addition to 20 μL/mL phosphatase, 20 μL/mL protease and 10 μL/mL EDTA (Pierce, Rockford, IL). The tube was then gently shaken for 5 minutes. The cells were then centrifuged at 14,000 x g for 10 minutes at 4 °C to pellet cellular debris. The resulting supernatant, containing all cellular proteins, was collected and stored at -20 °C until further analysis

Protein concentrations were determined using the Bradford (Sigma-Aldrich, USA) protein assay. Different masses of protein standards (25, 50, 100,150, 200 μg) were used to determine adequate amounts for detection of proteins. Lysates were diluted by 25% with sample loading buffer (PAGEgel, San Diego, CA) and 10% DDT reducer (PAGEgel). The samples were placed in boiling water for 3 minutes, and then samples were loaded into the wells of a pre-casted 10% SDS-PAGE western blot gel in duplicate (PAGEgel), the volume not to exceed 35 μL. In addition, purified protein samples were loaded onto the gel and served as controls. A prestained protein ladder, SeeBlue plus

(Invitrogen, USA) was used to insure transfer of protein to membrane. For the COX-2 protein standard (Oxford Biomedical Research, Oxford, MI) 1μg per lane was used, and for the MMP-3 protein standard (USBiological, Swampscott, MA) 30 μL per lane was used. The gels were run in 1 x PAGEgel SDS running buffer (PAGEgel) according to the protocols of PAGEgel 10x10cm SDS cassette gel running instructions, starting at 80 mA/gel or 160 mA for two gels. Current was decreased every 15 minutes by 20 mA, ending at a current of 70 mA for an approximate run time of 75-90 minutes. When the dye front reaches one inch from the bottom of the gel, it is transferred to a 0.2 μm nitrocellulose membrane (PAGEgel) using a constant current of 280 mA for 60 min while in western transfer buffer (Tris-Glycine-SDS Run/Blot Stock Buffer, PAGEgel) and 20% v/v methanol (Sigma-Aldrich). Following the transfer, the membranes were placed in blocking solution with TBST (western wash, femto TBST 10X (G-Biosciences, St. Louis, MO), 0.1% Tween-20 (Sigma-Aldrich, USA) and 5% nonfat milk (Nestle Carnation) for 1 h, then washed three times with TBST until the solution was clear. The membrane was incubated in primary antibody (mouse anti-human) overnight at 4°C. The primary antibodies were diluted to a concentration of 1:200 (COX-2), 1:100 (MMP-3), and 1:100 (control, β-Actin) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). All antibodies were diluted in TBST and 5% BSA. After incubation the primary antibody was removed and the membrane was washed with TBST three times for 5 min intervals. Then membranes are incubated in secondary antibody conjugated to a horseradish peroxidise (HRP) (1:2000) for 1 h at room temperature (goat anti-mouse, Santa Cruz Biotechnology Inc.). After the 1h incubation the secondary antibody was removed and the membrane was washed with TBST three times for 5 min intervals. Proteins were visualized on the

Typhoon multipurpose imager using Enhanced chemiluminescence ECL-plus® detection reagent (Amersham, Piscataway, NJ).

3.11 Semi-Quantitative RT-PCR

To confirm the data obtained through microarray analysis, semi-quantitative RT-PCR was performed. 100,000 (SD231RD2) cells were seeded in a T25 flask, maintained in MEM and 0.01 μM doxorubicin and 50,000 (MDA-MB-231) cells were seeded in a T-25 flask, maintained in MEM without drug for 72 h. RNA was extracted and converted into cDNA using QuantiTec Reverse Transcriptase KIT (QIAGEN, Valencia, CA) and following the manufactures protocol.

PCR was carried out by using ProMega Master Mix, (ProMega, Madison WI) and following manufactures protocol. To the Master mix, we added 10 μL of 5X Green Go Taq reaction buffer, 5μL of the cDNA product obtained from the Reverse Transcriptase Kit and 5 μL of each of the different primers (COX-2, MMP-3, Hep27, β-Actin) (QIAGEN, Valencia, CA). The cDNA is then placed in the Multistep III Thermostatic Circulator (Amersham Biosciences, Piscataway, NJ) and first denatured for 2 minutes at 95 °C. After the first denatured step the cDNA is amplified at 95 °C for 30 seconds, annealed at 55 °C for 45 seconds and the extension cycle was conducted for 60 seconds at 72 °C. This step of polymerization was repeated for 32 cycles. The final extension temperature is 72 °C for 5 minutes and then samples were kept at 4 °C until removed from instrument (Multistep III Thermostatic Circulator), then stored at -20 °C. The amplified products were then electrophoresed on 1 % agarose gels for 1h or until loading

dye reached the bottom of the gel. The gel is then stained in 0.5 μg/mL ethidium bromide (Sigma-Aldrich) and visualized with a UV illumination.

3.12 ELISA Determination of COX-2, MMP-3 and PGE_2

MDA-MB-231 (250,000) cells or SD231RD2 (400,000) were seeded into a 150 cm^2 flask maintained with a total volume of 20 mL of MEM cell culture medium. The resistant line (SD231RD2) was also supplemented with 400 μL of 0.0005 mM stock of doxorubicin, for a final concentration of 0.01 μM doxorubicin. After 5 days of incubation, the media was collected from the flask and put into 50 mL conical tube and centrifuged at room temperature for seven minutes at 1,500 rpm. The supernatant was collected and concentrated using a centrifuge filter device of 10-20 kDa (PALL). The adherent cells were rinsed three times with PBS, each rinse was discarded. Following the washing procedure, 5 mL of PBS was added to the flask and cells were scraped and transferred to a 15 mL conical tube. The cell solution was centrifuged at 1,500 rpm at room temperature for seven minutes and the resulting supernatant was discarded. The cells were lysed with 500 μL of cold RIPA cell lysis buffer (Assay Designs, Ann Arbor, MI), 20 μL/mL phosphatase, 20 μL/mL protease and 10 μL/mL EDTA (Pierce). The tube was placed on ice and then gently shaken for 5 minutes on a plate shaker. The cells were then centrifuged at 14,000 x g for 15 minutes at 4 °C, then supernatant was collected and stored at -20 °C until further analysis. To determine COX-2 expression, the cell lysate was used for COX-2 ELISA (Assay Designs, Ann Arbor, MI), according to manufactures instructions. To determine MMP-3 and PGE2, cell culture media was used for MMP-3

ELISA (Invitrogen, Carlsbad, CA) and PGE_2 ELISA (Assay Designs, Ann Arbor, MI), according to the manufacturer's instructions.

3.13 Cell Cycle Analysis

To determine cell cycle phase distribution following treatment of the six day assay, cells were harvested, and a cell pellet was obtained by centrifuging the cell solution at room temperature for seven minutes at 1,500 rpm. Cells were washed with 5 mL PBS, centrifuged and resuspended in 100 μL of PBS. To fix the cells, 1 mL of cold 95% ethanol was added slowly while shaking the sample gently. Samples were then stored at 4 ^{o}C for at least 24h before staining. To analyze the cells, propidium iodide staining was carried out. Fixed cells were washed with 2.0 mL of PBS and incubated with 0.1 mL Triton X 1% buffer and 0.1 mL RNase at 1 mg/mL for 10 min at room temperature. Propidium iodide stain (0.2 mL) at a concentration of 100 μg/mL was added and cells were incubated for 30 min in the dark at room temperature. Samples were analyzed on the Becton Dickinson FACSCalibur (Becton Dickinson, USA) and results were evaluated using ModFit LT Version 3.0 (verity Software House, Topsham, ME).

3.14 Live/Dead Viability Assay

. Following completion of the six day assay, cells were scraped in the maintenance media and the cell suspension was centrifuged for seven minutes at room temperature at 1,500 rpm. The supernatant was then discarded and the remaining cell pellet was washed with 5 mL of PBS. The washed pellet was centrifuged again, and the supernatant was discarded. The cell pellet was then re-suspended in 100 μL of PBS and 2 μL of the

working solution of SYTOX green (Molecular Probes, USA) and 2 μL of the working solution of C12 resaurzin (Molecular Probes, USA). Samples were then incubated for 15 min at room temperature, 200 μL of PBS was added, then placed on ice and analyzed immediately using the Becton Dickinson FACSCalibur (Becton Dickinson, USA) and results were evaluated using ModFit LT Version 3.0 (verity Software House, Topsham, ME).

3.15 Fluorescence Microscopy

On the sixth day of the six day viability assay, SD231RD2 and MDA-MB-231 cells were seeded into a 4-well chamber slide plate, with 40,000 cells/well and a total volume of 0.5 mL. The cells were incubated for 24h to allow cell density to reach 70 to 80% confluency. After 24 h media was removed and wells were washed with PBS and stained with 0.5 mL of Hoechst (2 μg/mL) and 0.5 mL of propidium iodide (10 μg/mL) diluted in PBS. The wells were incubated for 15 minutes and the images were acquired.

A bright field image was obtained visualizing cells with a phase lamp. Apoptotic cells were identified using a mercury lamp with an excitation wavelength of 300 -500 nm and an ultraviolet filter with an emission wavelength of 435-485 nm. Cells that have taken up the Hoechst stain are identified as apoptotic. Necrotic cells were identified using the mercury lamp and a green filter with an emission wavelength of 600-660 nm. A positive finding appeared red due to the presence of the propidium iodide stain within the cell. The images were taken with a Photometrics Cool Snap CCD camera attached to a Nikon Eclipse TE2000-U microscope (Nikon Inc. Melvile, NY) and analyzed using MetaVue software (Meta Series 6.0/6.1, Universal Imaging Corporation).

To determine the percent of apoptosis or necrosis, the following equation was used:

$$\frac{\text{Number of positive apoptotic/necrotic cells}}{\text{Total number of cells in the bright field}} \times 100\%$$

The three areas of the wells imaged were randomly selected and were scanned in each of the control and treated wells (n=3).

3.11 Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM), with groups consisting of three observations and each experiment preformed two or three times.

Determination of the Standard error of the mean: $\frac{\frac{stdev}{\sqrt{n}}}{average\ of\ the\ control}$

CHAPTER 4

FINDINGS OF THE STUDY

4.1 Analysis of Results

To determine the efficacy of doxorubicin and cisplatin on the breast cancer cell lines MDA-MB-231 and MCF-7, colony formation assays were performed to monitor proliferation. MCF-7 cells were exposed to a 1 h treatment of cisplatin at varying concentrations. The results of the survival gave a LD_{50} of 2.20×10^{-4} M for cisplatin (Figure 7A). By expanding the area between 100 and 1000 μM, a more precise LD_{50} was achieved. The expansion results in a LD_{50} of 4.27×10^{-4} M for cisplatin. Other LD_{50} values in our lab with cisplatin and MCF-7 with n=2 trials gave an LD_{50} of 3.13×10^{-4} M.

Doxorubicin survivals with MDA-MB-231 and MCF-7 are shown in Figure 8A and 8B, respectively. MDA-MB-231 was exposed to a 1 h treatment of doxorubicin at varying concentrations. The results of the survival gave an LD_{50} of 1.85×10^{-7} M for doxorubicin (Figure 8A). However, we find that MCF-7 cells exposed to varying concentrations for 1 h are slightly less sensitive to doxorubicin with an LD_{50} of 6.85×10^{-7} M (Figure 8B). MBA-MD-231 clonogenic survivals using cisplatin were not performed, because their LD_{50} values were determined previously by Van Vo to be 3.33×10^{-4} M (21).

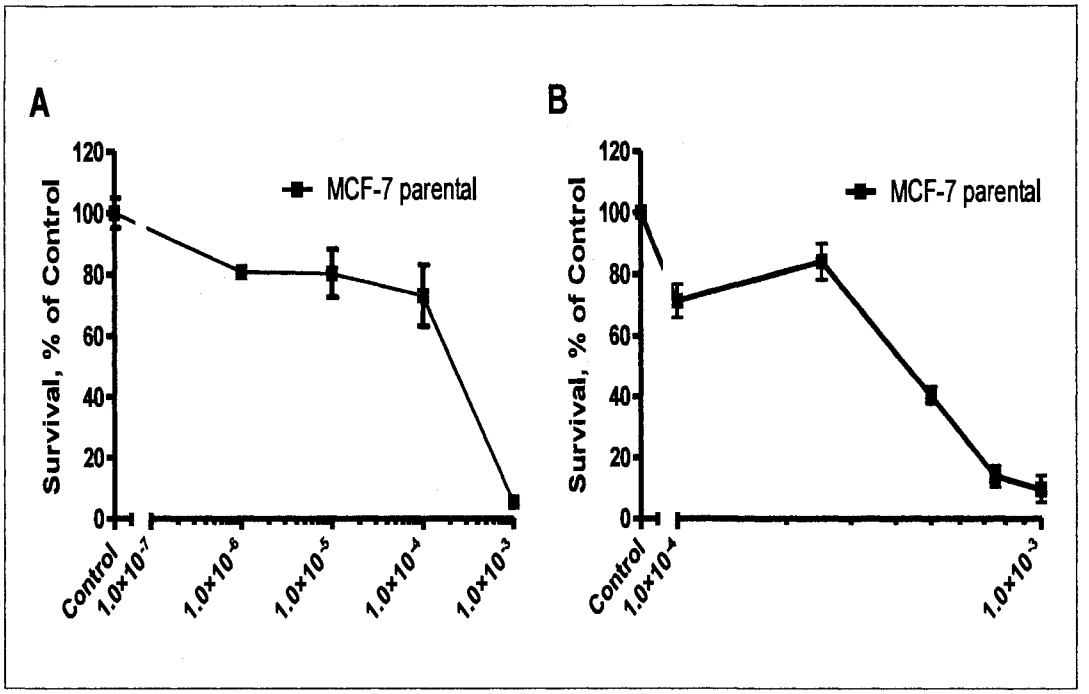

Figure 7. Clonogenic survival with 1h treatment cisplatin and MCF-7. Cell survival was measured and compared to control cells after a 1 hour exposure to varying concentrations of cisplatin (M). The above figure A and B represents one trial each. Colonies were stained with crystal violet and viability was measured by counting colonies and comparing to the number of colonies in the control sample. A colony is a group of cells with $n \geq 50$.

Determining the LD_{50} of these drugs helped distinguish which concentrations should be used to develop the resistant cell lines. The most appropriate concentrations to begin with were two concentrations that were above the LD_{50}, a value close to the LD_{50} and two concentrations below the LD_{50}. Preparation of the MDA-MB-231 resistant cell line required six months of continuous exposure to 10^{-8} M doxorubicin; the resistant cell line is referred to as SD231RD2. The naming convention utilizes the researcher's initials, SD; a portion of the wild type cell line nomenclature, 231 from the full name MDA-MB-231; an indication of resistance to doxorubicin, RD; and the colony number harvested,

specifically "2" for the second colony harvested from the population that was exposed to continuous treatment.

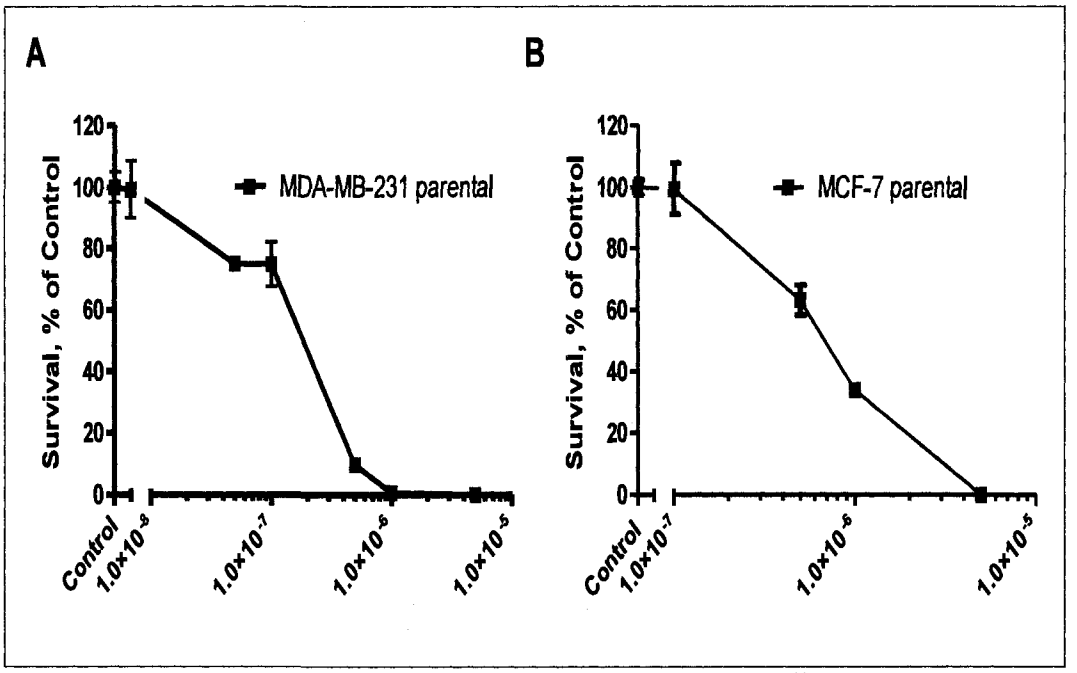

Figure 8. Clonogenic survival with 1hr treatment doxorubicin. Cell survival was measured and compared to control cells after a 1 hour exposure to varying concentrations of Doxorubicin (M). Figure A represents three trials for MDA-MB-231, whereas Figure B represents one trial for MCF-7. Colonies were stained with crystal violet and viability was measured by counting colonies and comparing to the number of colonies in the control sample. A colony is a group of cells with n=50 or greater.

The MCF-7 resistant cell line prepared also required approximately six months of continuous exposure to 10^{-7} M, 10^{-6} M and 10^{-4} M cisplatin. Although more than one resistant line was produced with cisplatin, the naming convention described previously is applicable to this cell line with M7 representing the original cell line nomenclature, RC indicating resistance to cisplatin and the final number indicated the colony harvested from the population that was exposed to continuous treatment. The resistant cell lines

distinguished as SDM7-RC16 through SDM7-RC22 are colonies derived from 10^{-7} M concentrations of cisplatin exposure; SDM7-RC9 through SDM7-RC15 were lines developed from exposure to 10^{-6} M concentrations of cisplatin exposure and SDM7-RC7 and SDM7-RC8 were lines developed from exposure to 10^{-4} M concentrations of cisplatin exposure (Table 1).

Resistant Cell Line	Resistant to Anticancer Agent	Proliferation Status
SDM7-RC7 & SDM7-RC8	100 μM Cisplatin	Proliferation Ceased
SDM7-RC9 – SDM7-RC15	1 μM Cisplatin	Proliferation Ceased
SDM7-RC16-SDM7-RC22	0.1 μM Cisplatin	Proliferation Ceased
SD231RD2	0.01 μM Doxorubicin	Ongoing Proliferation
SDM7-RD	0.01 μM Doxorubicin	Never Developed
SD231RC	1 μM Cisplatin	Never Developed

Table 1. Report of resistant cell lines produced and proliferation status. Table indicates the resistant cell line name, concentration of anticancer agent the line is resistant too and also reports if the cell line is still proliferating or if the cell line is not viable. The cell lines in which the proliferation ceased was due to a change in fetal bovine serum.

To produce evidence to support whether the cell lines are resistant by increased viability of the resistant line when compared to wild type, AlamarBlue, clonogenic survivals, and 6 day viability assays were performed. AlamarBlue was the first test preformed. The first resistant cell line produced SDM7RC had many clones produced with MCF-7 and cisplatin at 10^{-4}, 10^{-6} and 10^{-7}M.

The prediction was that the 10^{-4} colonies would be more resistant to cisplatin when compared to wild type, followed by 10^{-6} and 10^{-7} colonies. However, when tested with AlamarBlue we did not see a trend. At the highest cisplatin concentration (1000 μM) the wild type cell line demonstrated a percent survival of 29.91 ± 1.22 %. The resistant lines at 10^{-4}M cisplatin, SDM7-RC7 and SDM7-RC8, produced survivals of 33.54 ±1.09 and 28.16 ± 0.41 %, respectively. SDM7-RC14 & RC15 at 10^{-6}M cisplatin (Figure 9) and SDM7-RC9 & RC13 (Figure 10) produced an increase in survival of approximately 20% when compared to wild type. Both cell lines at 10^{-7} produced an increase of survival of 10% (Figure 9-10).

Comparing the resistance capabilities of each cell line that were predicted to be resistant to the same concentration of drug revealed that not all lines are equally resistant to cisplatin. SDM7-RC18 and SDM7-RC17 are not resistant to 10^{-7} M cisplatin, while SDM7-RC19 and SDM7-RC20 are slightly resistant by 10% (Figure 10).

When the duplicate test was performed one month later, the resistant capabilities of the cell lines diminished slightly. RCM7-RC15 and RCM7-RC9 survival was only 10% higher than wild type (Figure 11). These resistant lines were also starting to diminish slowly and were not proliferating at their rates previously observed.

Clonogenic survivals were preformed to test the sensitivity of AlamarBlue and to confirm if the resistant MCF-7 cell lines were truly demonstrating resistance. SDM7-RC13 and SDM7-RC14 demonstrate a higher sensitivity to the cisplatin then wild type, clearly indicating no resistance (Figure 12). SDM7-RC7 and SDM7-RC20 show a 20% higher resistance when cells are treated with a concentration of 100 μM cisplatin compared to wild type. When exposed to higher concentrations of 1000 μM cisplatin, all

cell lines responded the same way and produced no colonies. The resistant MCF-7 cell lines stopped proliferating and died out completely due to the use of a different manufacturer's fetal bovine serum. Therefore, no further tests were performed.

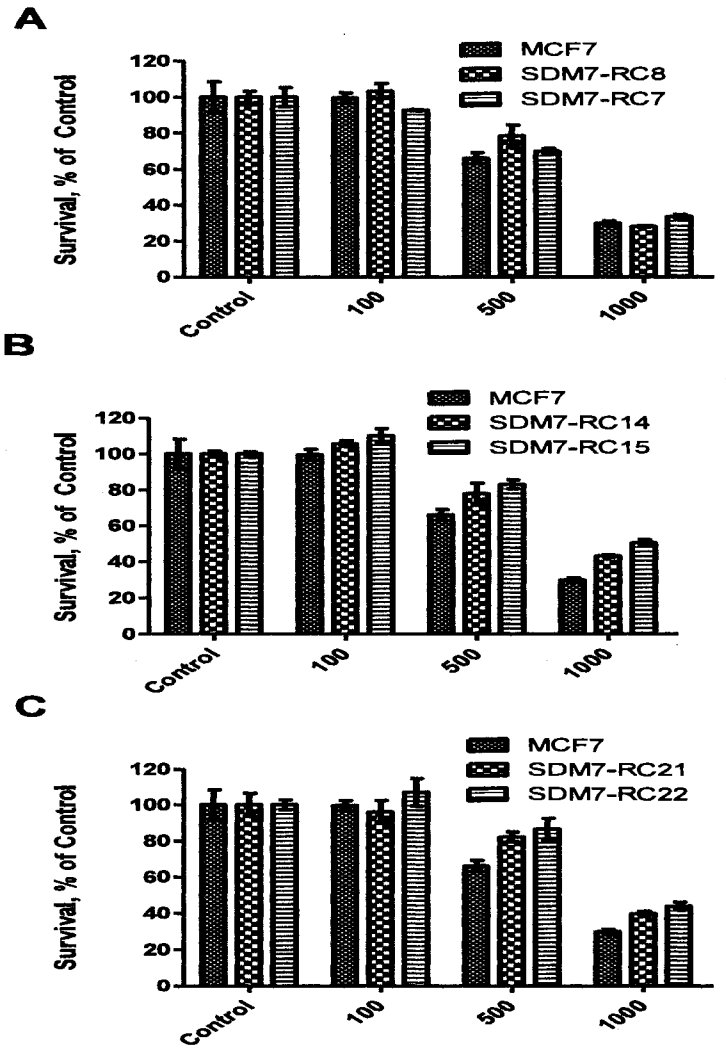

Figure 9. AlamarBlue assay using wild type MCF-7 and resistant cell lines. Cell survival was measured and compared to control cells after a 24 h exposure to 100, 500 and 1000 μM cisplatin. Figure A represents resistant lines from 10^{-4}M cisplatin, B represents resistant lines from 10^{-6}M cisplatin and C represents resistant lines from 10^{-7}M cisplatin. Cell viability was measured by staining cells with AlamarBlue and measuring absorbance at 595nm using a spectrophotometer. n=1 trial and triplicate readings with SEM.

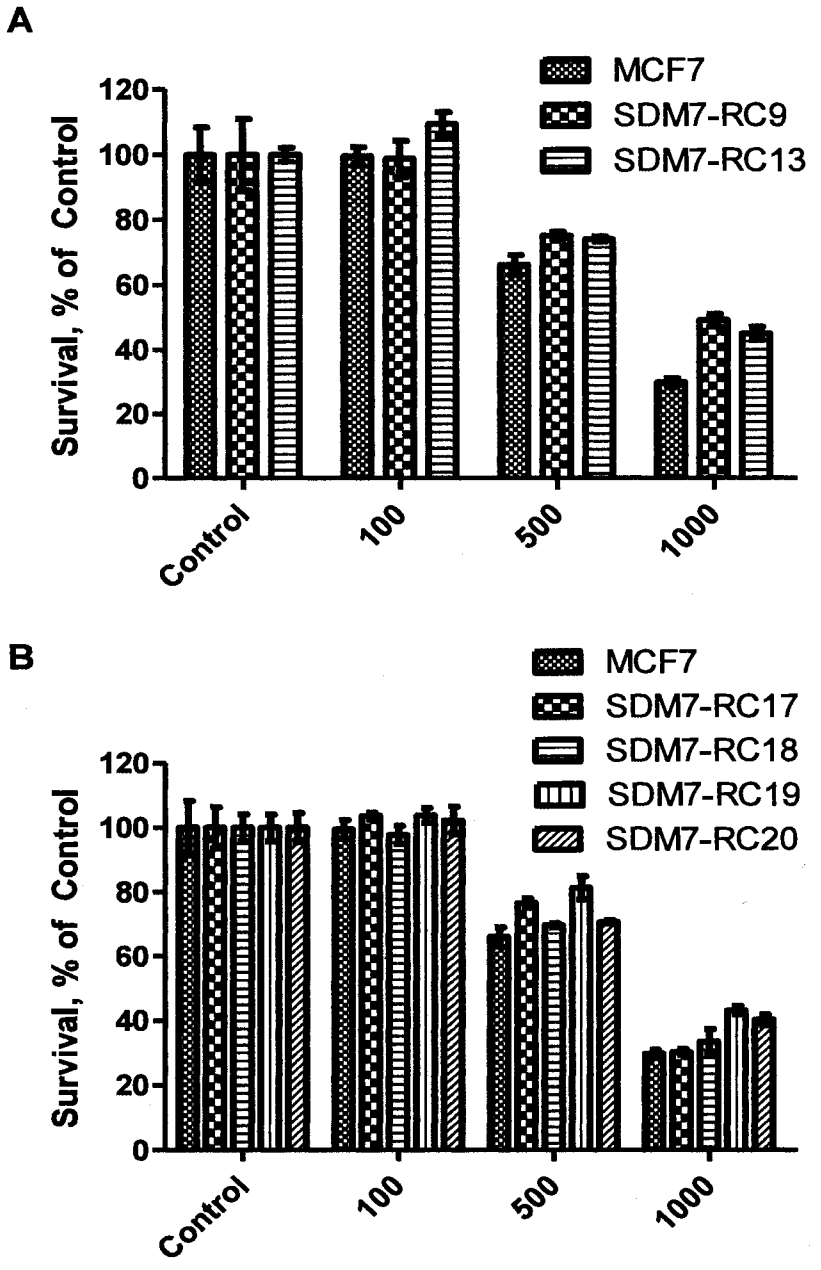

Figure 10. Alamar Blue Assay using wild type MCF-7 and resistant cell lines. Cell survival was measured and compared to control cells after a 24 h exposure to 100, 500 and 1000 μM cisplatin. Figure A represents resistant lines from 10^{-6}M cisplatin and B represents resistant lines from 10^{-7}M cisplatin. Cell viability was measured by staining cells with AlamarBlue and measuring absorbance at 595nm using a spectrophotometer. n=1 trial, and triplicate readings with SEM.

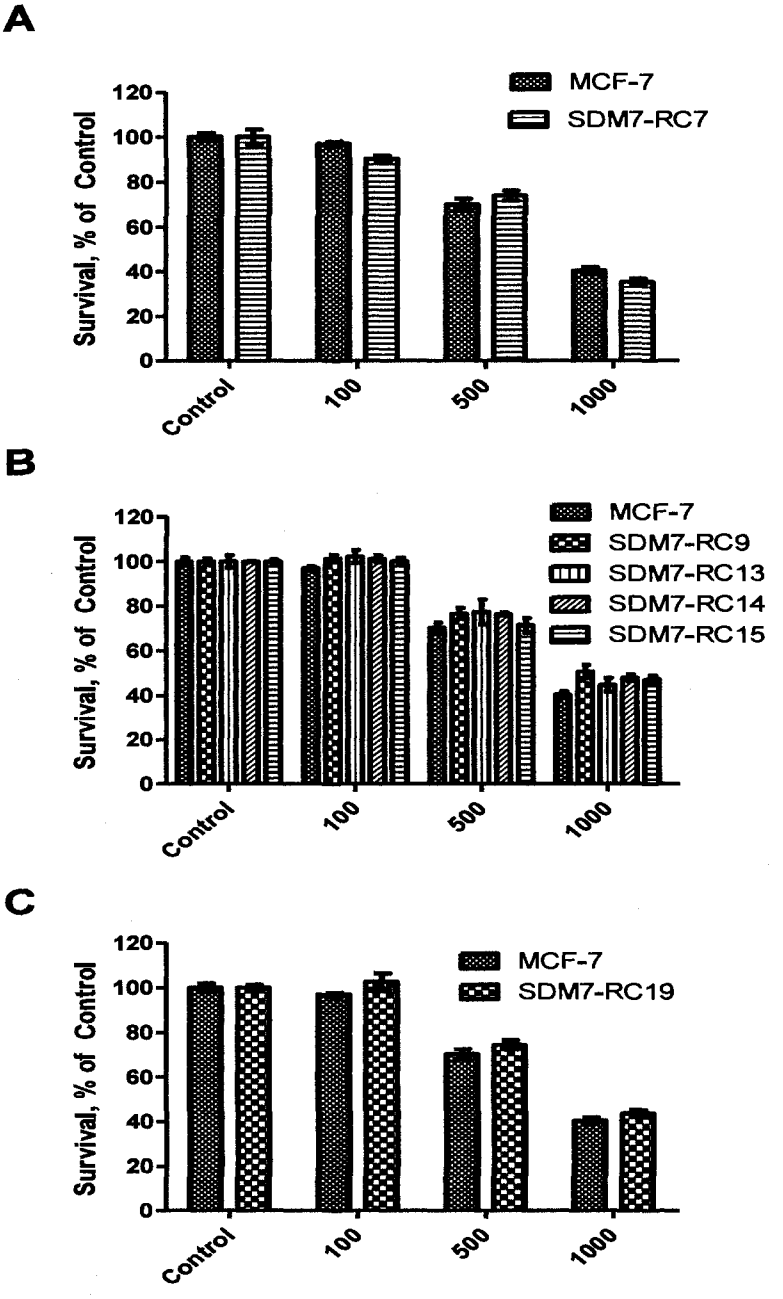

Figure 11. AlamarBlue assay using wild type MCF-7 and resistant cell lines. A duplicate cell survival was measured and compared to control cells after a 24 h exposure to 100, 500 and 1000 μM cisplatin. Figure A represents resistant lines from 10^{-4}M cisplatin, B represents resistant lines from 10^{-6}M cisplatin and C represents resistant lines from 10^{-7}M cisplatin. Cell viability was measured by staining cells with AlamarBlue and measuring absorbance at 595nm using a spectrophotometer. n=1 trial, and triplicate readings with SEM.

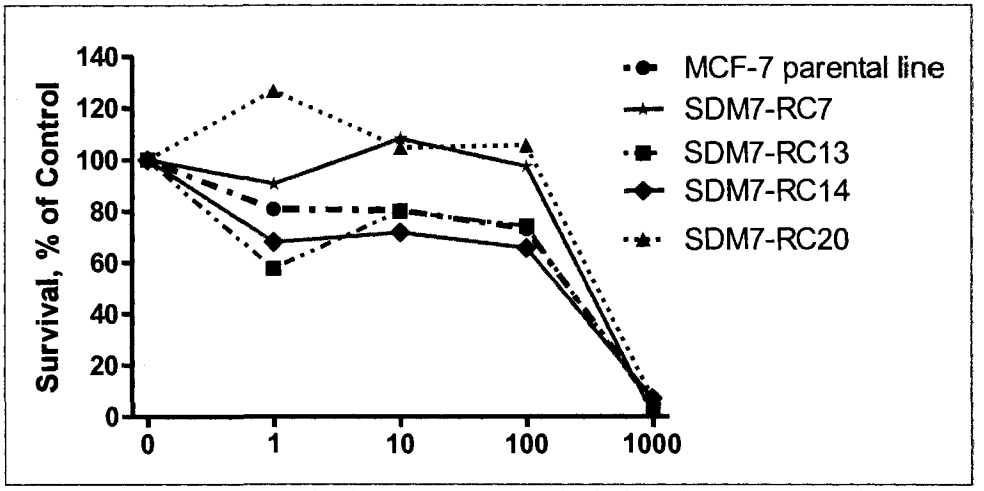

Figure 12. Clonogenic survival with 1h treatment cisplatin using MCF-7 resistant cell lines. Cell survival was measured and compared to control cells after a 1 h exposure to varying concentrations of Cisplatin (μM). The above figure represents one trial. Colonies were stained with crystal violet and viability was measured by counting colonies and comparing to the number of colonies in the control sample. A colony is a group of cells with n=50 greater.

The last resistant line, developed from the parental line MDA-MB-231, which was exposed to 10^{-8} M doxorubicin. We tested drug exposure for 24 h and 48 h using AlamarBlue assay, and found SD231RD2 slightly more resistant to exposure to doxorubicin at both 24 and 48 h. At all drug concentrations tested, SD231RD2 was more resistant by approximately 10% when compared to wild type (Figure13).

To confirm resistance clonogenic survivals were used, which is a more sensitive assay in comparison to AlamarBlue assay. Wild type MDA-MB-231 and SD231RD2 were exposed to 1h treatments of doxorubicin, the wild type survival is shown in Figure 8 and the resistant line survival is shown in Figure 14. The LD_{50} of SD231RD2 was found to be 3.75×10^{-8} M (0.0375 μM). This value is compared to the parental who gave an LD_{50} of 1.85×10^{-7} M doxorubicin (0.185 μM). Since this survival did not confirm resistance, exposure time of doxorubicin was increased to 24 h for both the resistant and wild type

cell line (Figure 15). With the 24 h exposure to doxorubicin, we see a 5 fold difference in the LD_{50} values. Wild type is more sensitive to doxorubicin at a 24 h exposure (LD50 of 1.16 $x10^{-8}$ M) while SD231RD2 is more resistant with an LD50 of 5.58 x 10^{-8} M.

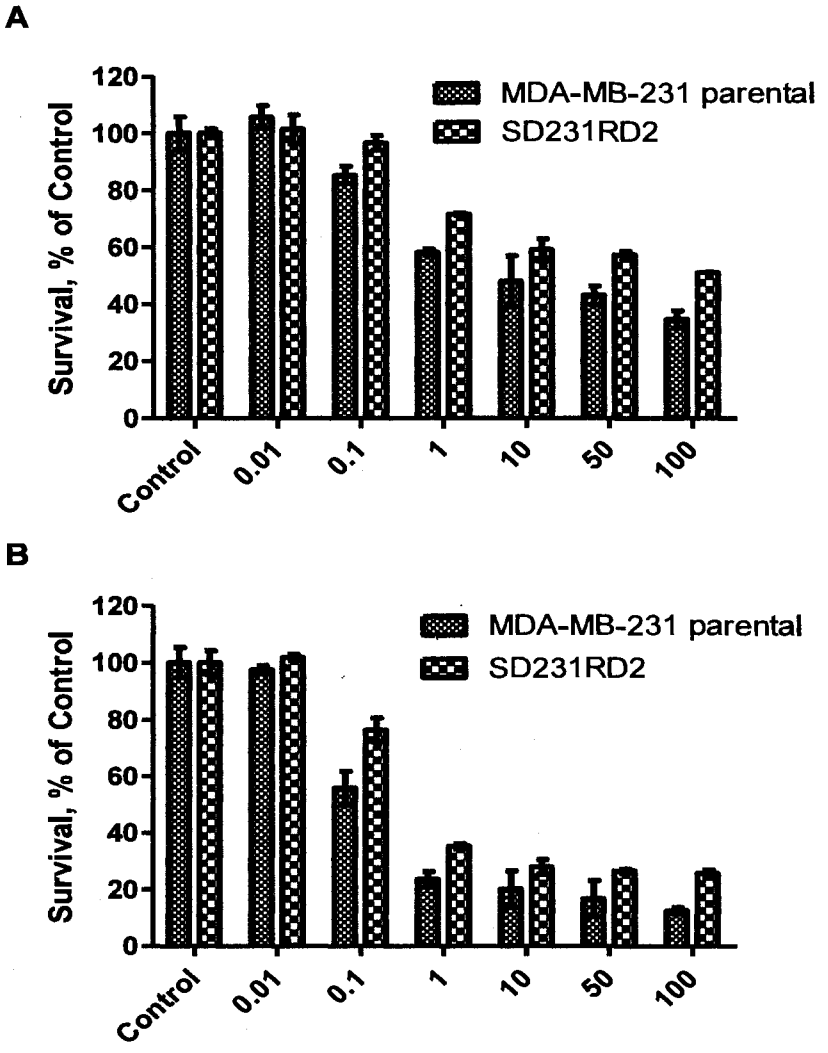

Figure 13. AlamarBlue assay using wild type and resistant MBA-MD-231 cell lines. Cell survival was measured and compared to control cells after a 24 (Figure A) and 48 hour exposure (Figure B) to varying concentrations of doxorubicin (μM). Cell viability was measured by staining cells with AlamarBlue and measuring absorbance at 595nm using a spectrophotometer. The figure represents two trials and triplicate readings with SEM.

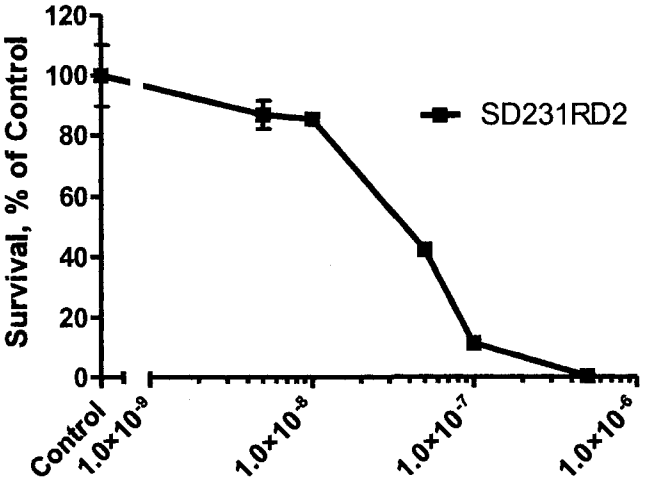

Figure 14. Clonogenic survival with 1 h treatment doxorubicin, using SD231RD2. Cell survival was measured and compared to control cells after a 1 hour exposure to varying concentrations of doxorubicin (M). The figure represents one trial. Colonies were stained with crystal violet and viability was measured by counting colonies and comparing to the number of colonies in the control sample. A colony is a group of cells with n=50 greater.

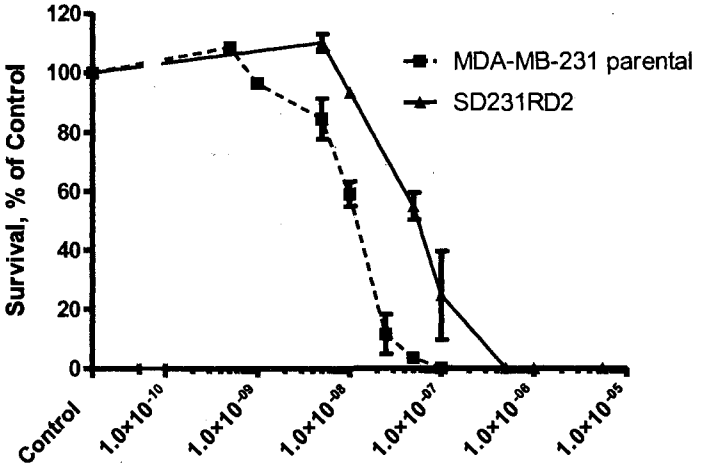

Figure 15. Clonogenic survival with 24 h treatment doxorubicin, using MDA-MB-231 and SD231RD2. Cell survival was measured and compared to control cells after a 24 hour exposure to varying concentrations of Doxorubicin (M). The figure represents three trials. Colonies were stained with crystal violet and viability was measured by counting colonies and comparing to the number of colonies in the control sample. A colony is a group of cells with n=50 greater.

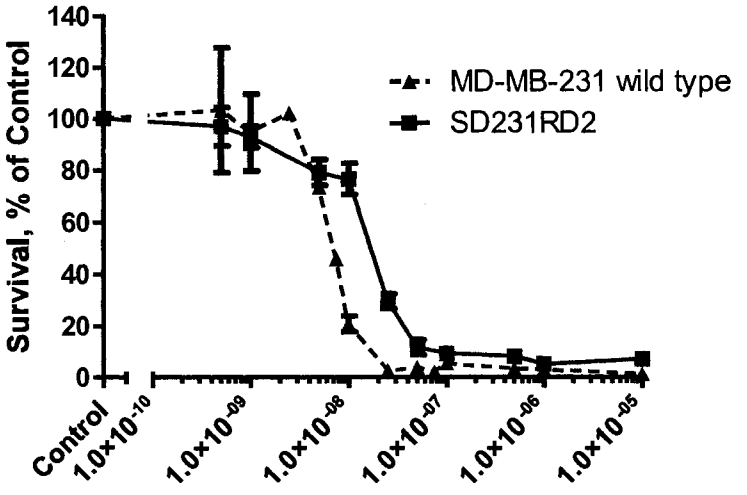

Figure 16. Six Day Viability Assay using Doxorubicin. MDA-MB-231 wild type and resistant cell line SD231RD2 were exposed to a 6 day exposure of varying concentrations of doxorubicin (M). Cell survival was measured by counting cells using a coulter counter and comparing to control cell numbers. The figure represents 3 trials, of triplicate readings.

One more assay was performed prior to microarray analysis. The six day assay confirmed that the cell line produced was indeed resistant with a 60% difference in survival rates when both the resistant cell line and the parental were exposed to a concentration of 0.01 μM doxorubicin (Figure 16).

To further analyze the extent of resistance, resistant and wild type cells were either treated or not treated with chemotherapeutic agent for six days and analyzed using the live/dead viability assay, phase cycle analysis and fluorescence microscopy. For the live/dead assay we hoped to see a significant increase in cell viability for the resistant line when compared to the wild type. Figure 17 shows the percentage of cells that are viable, injured (which indicates the first step of apoptosis) or dead. The wild type had approximately 20% less viable cells compared to the resistant line with or without drug,

and when compared to the wild type line without chemotherapeutic agent. The resistant line had a slight response within error to the chemotherapeutic agent when compared to control cells of both the resistant line and wild type lines.

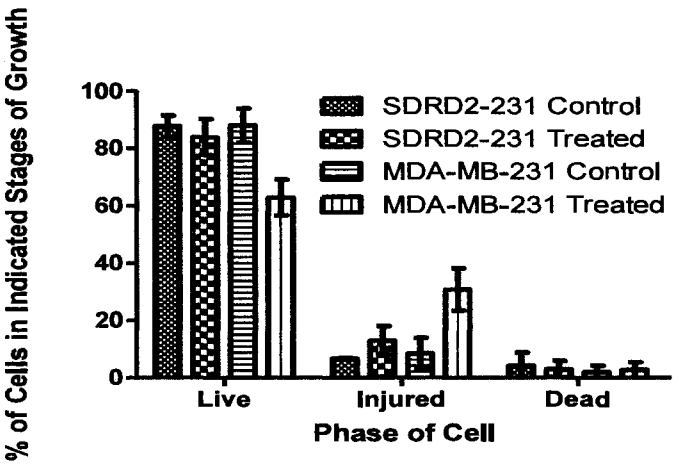

Figure 17. Live/Dead Viability Assay with SD231RD2and MDA-MB-231 cells. Cells were harvested on day 6 of the Six Day Viability Assay. The figure is representative of 2 trials. The study demonstrates that after six days of continuous exposure to doxorubicin, the wild type cell line experiences a higher percentage of injured cells, which is indicative of apoptosis. The resistant line demonstrates a slight increase in the number of injured cells in comparison resistant line control which was not exposed to chemotherapy treatment.

In another flow cytometry experiment, phase cycle analysis, cells were collected on day six of a six day exposure to doxorubicin. The wild type cell line, MDA-MB-231 which was treated for six days, displayed a block in G2/M phase when compared to the untreated line. This is evident by a decrease in G0/G1 and S phases, which leads to the subsequent increase in G2/M phase (Figure 18A). On the other hand, there was no block in the any phase of the cell cycle seen for the resistant treated cell line, SD231RD2 when compared to both the resistant line that was untreated (Figure 18B) and the parental wild

type line MDA-MD-231. The apoptotic peak had an increase of 20% in the treated wild type when compared to wild type control and resistant lines.

Fluorescence microscopy was used to distinguish between an apoptotic or necrotic mechanism of death. Again cells were harvested on day 6 after exposure to 0.01 μM doxorubicin for six days, seeded, and incubated for 24 h. Cells were stained with Hoechst and propidium iodide to visualize apoptotic and necrotic cells. The wild type line indicated that the mechanism of death is apoptotic due to the substantial increase in apoptotic cells upon treatment with the drug (Table 2). However, this trend is not observed with necrosis. There was no increase in necrotic cells when compared to control. The resistant cell line did not point to a specific mechanism of death, but there were more apoptotic cells than necrotic. Microscopic images of the cells can be seen in Figure 19A, show the differences in the wild type line that is untreated and treated for 6 days. The images clearly demonstrate the effects of 6 day exposure by a reduced number of cells and illustrate changes in the morphology of the wild type cell line (Figure 19B). The resistant line did not demonstrate the same trend. The resistant line when exposed to no drug (Figure 19C) appears to have no morphological changes when compared the resistant line that was exposed to drug (Figure 19D). Also, there were no differences in cell proliferation of the exposed resistant line when compared to control (non-treated resistant). It should also be pointed out that the parental line (not exposed to drug) and resistant line (exposed or unexposed) did not appear to have a difference in morphology.

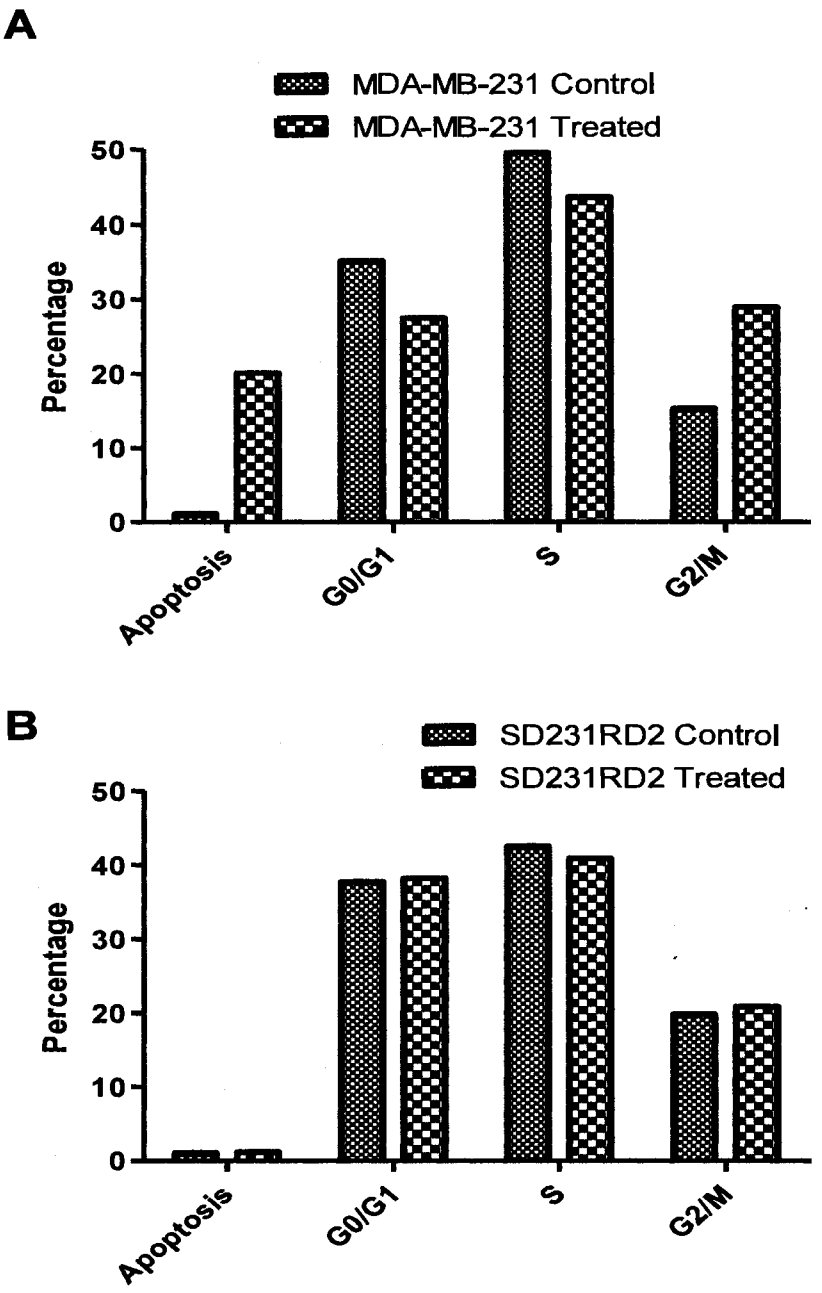

Figure18. Cell cycle analysis of MDA-MB-231 and SD231RD2. Cells were harvested after a six day exposure to 0.01 μM doxorubicin than analyzed by flow cytometry. The wild type cell line demonstrates a block in the G2/M phase (A), while the resistant line SD231-RD2 does not experience a block in any phase of the cell cycle when compared to control resistant cells that were not exposed to drug (B). The figure represents one trial.

Treatment	Percent Apoptosis	Percent Necrosis
MDA-MB-231 Control	1.7 ± 0.6	0.9 ± 0.2
MDA-MB-231 6 Day Exposure (DOX)	16.9 ± 4.5	2.0 ± 2.3
SD231RD2 Control	4.3 ± 0.8	0.9 ± 0.1
SD231RD2 6 Day Exposure (DOX)	5.9 ± 0.1	1.1 ± 0.8

Table 2. Fluorescence microscopy analysis of MDA-MB-231 and SD231RD2 cells. Cells were harvested after a six day exposure to 0.01 μM doxorubicin than analyzed by flow cytometry. The wild type cell line demonstrates a significant increase in apoptosis. While the resistant line SD231-RD2 also follows an apoptotic mechanism of death. The data in the table is representative of two independent trials.

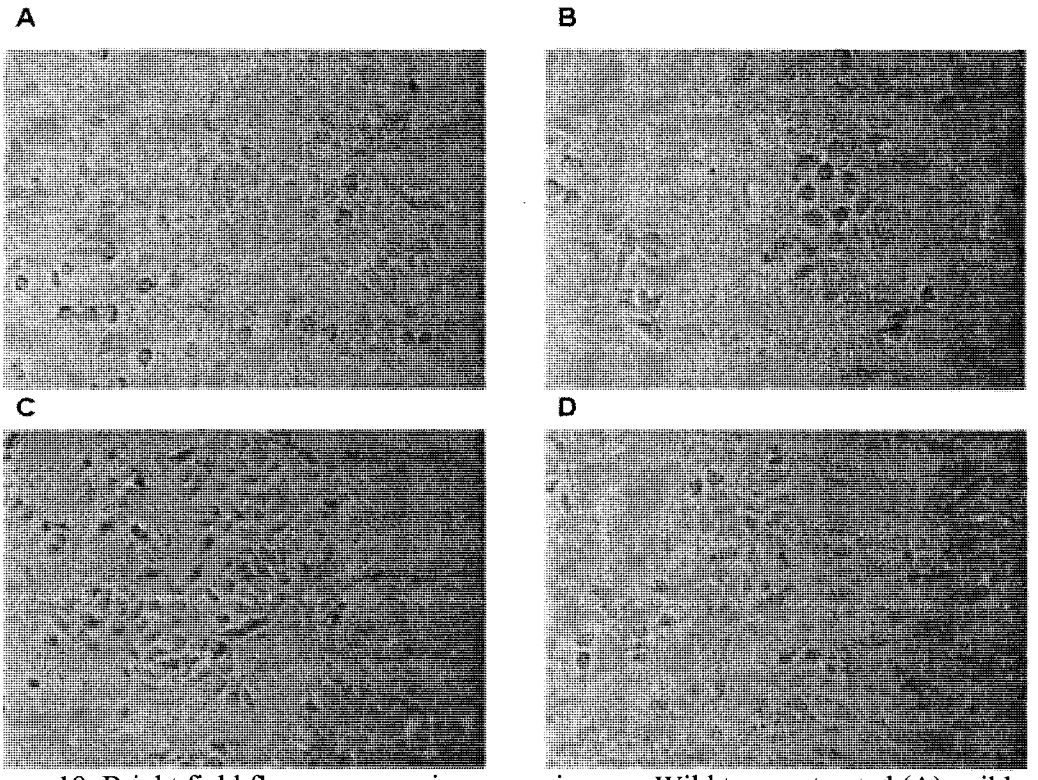

Figure 19. Bright field fluorescence microscopy images. Wild type untreated (A), wild type treated (B), resistant line untreated (C) and resistant line treated (D) by six day exposure to doxorubicin, harvested and incubated for 24 h, and then images were obtained.

The data supports that the resistant cell line produced is resistant to doxorubicin, therefore samples of RNA were sent to the Winber Institute for wild type and resistant lines analysis of gene expression. Genes that were more highly expressed are shown in Table 3. The genes of interest were COX-2 and MMP-3, which had fold changes of 11.96 and 14.93, respectively, in comparison to wild type gene expression levels.

To confirm the microarray data, Western blot analysis was performed to establish a baseline for expression of our proteins of interest. The first attempt with western analysis used 40, 60 and 80 μg of protein, and our results indicated that neither COX-2 nor MMP-3 was present (Figure 20). For the second trial, the protein mass was increased to 120 and 150 μg and protein standards COX-2 (2 μg), MMP-3 and β-Actin were incorporated. However, the presence of the proteins was not established and the protein standard for COX-2 was too strong, which is indicated by the thick band (Figure 21). Also, MMP-3 standard was not observed. The final attempt used protein masses of 150 and 200 μg of protein, also a lower concentration for protein standard COX-2 (0.5 and 1 μg) and for MMP-3 fresh antibody was made at the strongest concentration. Both protein standards were observed along with β-Actin, but the proteins of interest from the cell lysates were still not observed (Figure 22). Since there was no protein expression in western analysis, a confirmation of the microarray analysis is needed to ensure overexpression of the mRNA. Therefore semi-quantitative PCR was performed. The PCR data (Figure 23) confirmed the over-expression levels of the COX-2, MMP-3 and Hep27 (protein not of interest, but still overexpressed). A repeat of PCR was ran in the presence of a base pair ladder, this is shown in Figure 24. In order to justify the PCR adducts to be valid, loading controls such as β-actin and a smaller base pair ladder of 50-200 was used (Figure 25).

Gene Names (Common)	Fold Change	Activity	Protein Expression
COX-2 prostaglandin-endoperoxide synthase 2	11.96	anagen blood pressure regulation cell motility cyclooxygenase pathway fatty acid biosynthetic process keratinocyte differentiation prostaglandin biosynthetic process regulation of inflammatory response	cytoplasm membrane nucleus
MMP-3 matrix metalloproteinase 3 (stromelysin 1, progelatinase)	14.93	collagen catabolic process peptidoglycan metabolic process proteolysis	extracellular space proteinaceous-extracellular matrix
TRIP14 2-5oligoadenylate synthetase-related protein p30 (OASL)	17.55	biological_process immune response protein modification process	cytoplasm nucleolus
HEP27; DHRS2 short-chain alcohol dehydrogenase	18.94	C21-steroid hormone metabolic process electron transport metabolic process negative regulation of progression through cell cycle	nucleus
H2A/l H2A histone family	19.03	chromosome organization and biogenesis nucleosome assembly	chromosome nucleosome nucleus
G10P2	27.21	biological_process immune response	cellular_component
H2B/b H2B histone family	36.66	chromosome organization and biogenesis nucleosome assembly	chromosome nucleosome nucleus

Table 3. Microarray analysis. MDA-MB-231 wild type cells were compared by microarray analysis to the resistant line, SD231RD2. The above table shows the ten genes that had the largest increase in expression in SD231RD2 cells in comparison to MDA-MB-231 wild type cells. The Table also indicates function of the protein, the activity it possesses and where the protein can be found in the cell.

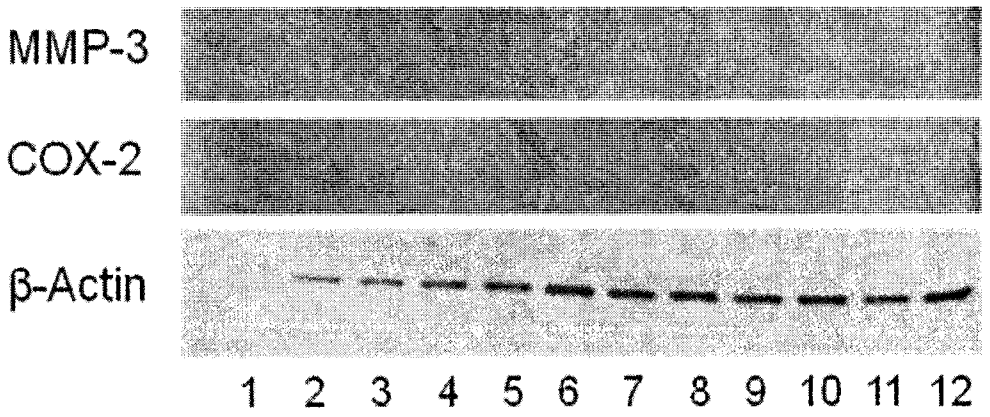

Figure 20. Western blot analysis of MDA-MB-231 and SD231RD2 lines. Western blots were looking for the production of COX-2 and MMP-3 using protein concentrations of 40, 60 and 80 μg. Lane one is transfer ladder, followed by wild type cell 40, 60 and 80 μg of protein from left to right in lanes 2-7 duplicate. The next 8-12 lanes are SD231RD2 with 40, 60 and 80 μg of protein from left to right in duplicate (80 μg for the resistant line was loaded only once in lane 12). β-Actin is used as a loading control.

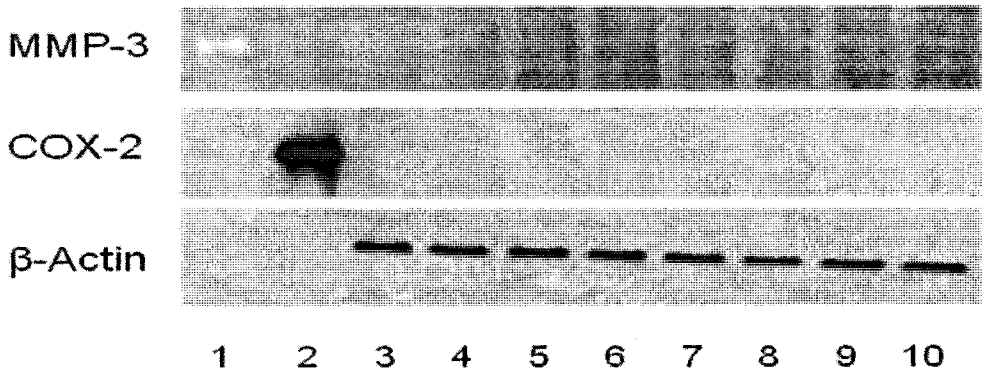

Figure 21. Western blot analysis of MDA-MB-231 and SD231RD2 lines. Western blots were looking for the production of COX-2 and MMP-3 using protein masses of 120 and 150 μg. Lane one is transfer ladder, lane two is either COX-2 or MMP-3 protein standard. MDA-MB-231 with 120 μg of protein (lanes 3-4) and 150 μg of protein (lanes 5-6) from left to right in duplicate. SD231RD2 with 120 μg of protein (lanes 7-8) and 150 μg of protein (lanes 9-10) from left to right in duplicate. β-Actin is used as a loading control.

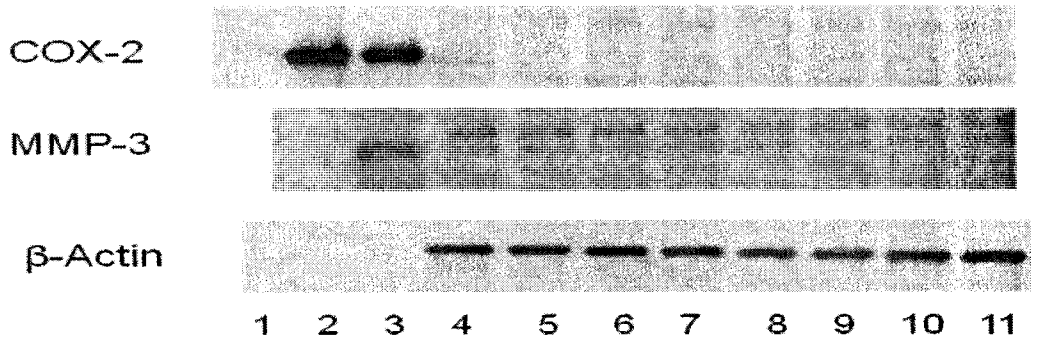

Figure 22. Western blot analysis of MDA-MB-231 and SD231RD2 lines. Western blots were used to determine production levels of COX-2 and MMP-3 using protein masses of 150 and 200 μg. Lane one or two is transfer ladder, followed by COX-2 (lane 2-3) or MMP-3 (lane 3) protein standard. MDA-MB-231 with 150 μg (lanes4-5) and 200 μg (lanes 6-7) of protein from left to right in duplicate. SD231RD2 with 150 μg (lanes 8-9) and 200 μg (lanes 10-11) of protein from left to right in duplicate. β-Actin is used as a loading control.

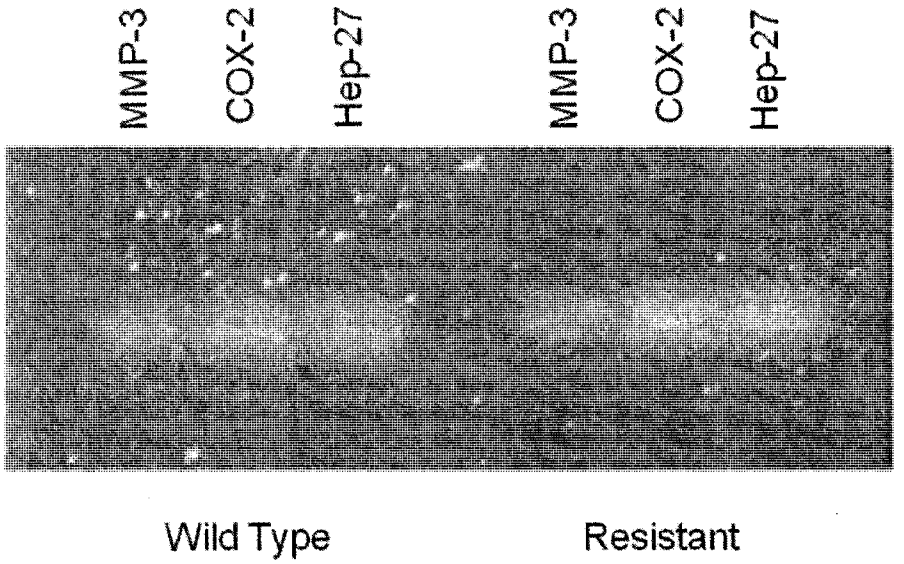

Figure 23. Semi-Quantative PCR of wild type and resistant cell lines. PCR confirmed presence of mRNA products of MMP-3, COX-2 and Hep27.

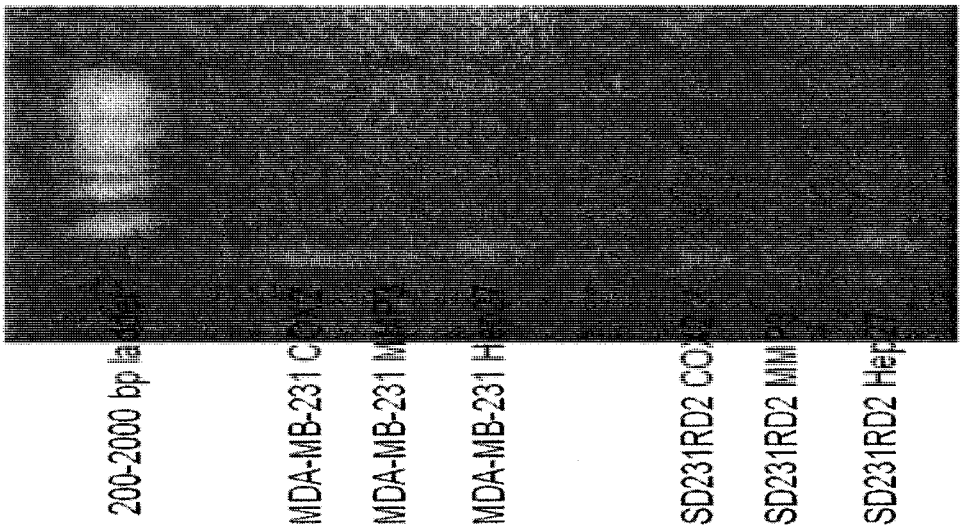

Figure 24. Semi-Quantative PCR of wild type and resistant cell lines. PCR confirmed presence of mRNA products of MMP-3, COX-2 and Hep27. Lane one is a 200-2000 base pair ladder, lanes are identified in figure.

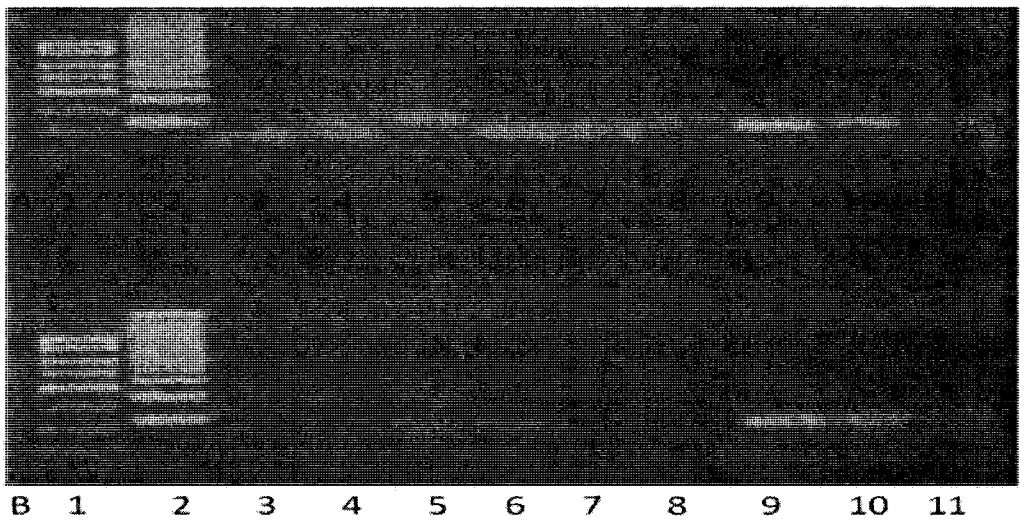

Figure 25. Semi-Quantative PCR of wild type and resistant cell lines. PCR confirmed presence of mRNA products of MMP-3, COX-2 and β-actin. Gel A detection of COX-2, lane 1 is a 50-200 base pair ladder, lane 2 is a 200-2000, lanes 3-5 are MDA-MB-231 in volumes of 2.5, 5 and 10 μL of cDNA. Lanes 6-8 are SD231RD2 in volumes of 2.5, 5 and 10 μL of cDNA, lanes 9-11 is loading control β-actin for SD231RD2 in volumes of 2.5, 5 and 10 μL of cDNA. Gel B, lanes are identical but detecting MMP-3 instead of COX-2. Lanes 9-11 are loading control β-actin for MDA-MB-231 in volumes of 2.5, 5 and 10 μL of cDNA.

Also, figure 25 used fresh isolated RNA from SD231RD2 to ensure that the resistant lines RNA was not changing with time, to eliminate the factor of variance in the genome.

To examine lower concentrations of the proteins COX-2 and MMP-3, ELISA assays were performed. Figures 26 and 27 illustrate a typical protein standard curve for COX-2 and MMP-3, respectively. From the standard curves the results indicate that there is slight increase of both these proteins in the resistant lines compared to the wild type. There was no indication of COX-2 present in the wild type cell line, while there was a slight amount of COX-2 (2 ng/mL) present in the resistant line. The amount of MMP-3 present in SD231RD2 was found to be 0.33 ng/mL and the concentration of MMP-3 in the parental line which was 0.059 ng/mL (Table 4). However, all these concentrations are below the level of detection in the kit.

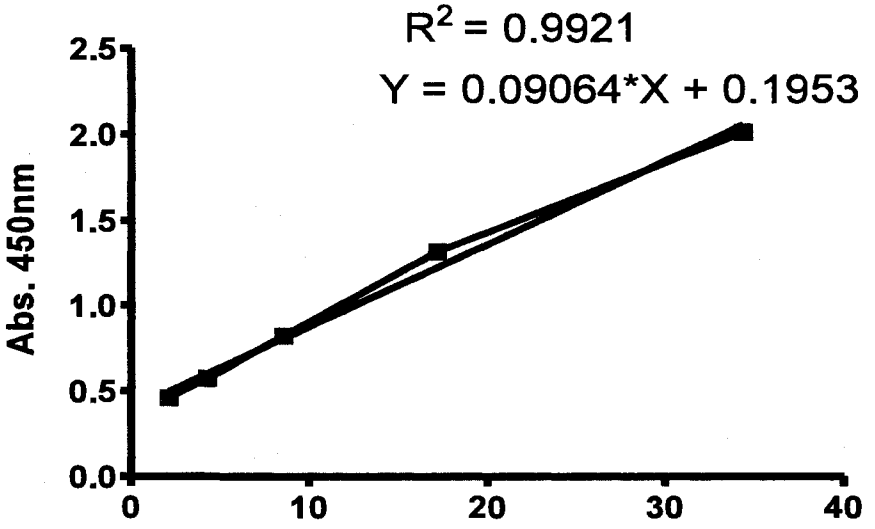

Figure 26. ELISA standard protein curve for COX-2. The y-axis represents Absorbance at 450 nm and the x-axis represents concentration of protein in ng/mL. The unknown samples are determined by the linear regression line shown on the graph.

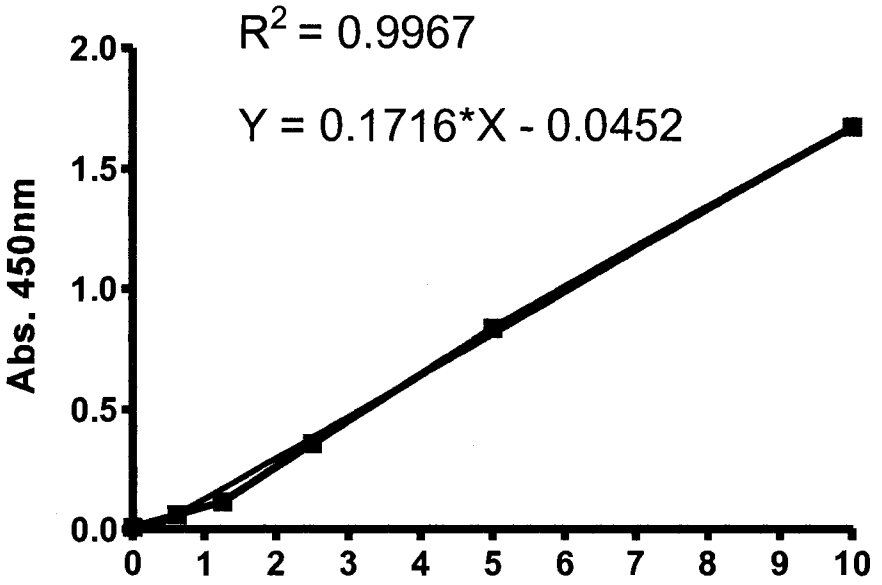

Figure 27. ELISA standard protein curve for MMP-3. The y-axis represents Absorbance at 450 nm and the x-axis represents concentration of protein in ng/mL. The unknown samples are determined by the linear regression line shown on the graph.

To further analyze these results, we decided to try to inhibit COX-2 even though we were not seeing the protein in western analysis or ELISA data. On inhibition on COX-2 with 50μM of indomethacin the LD_{50} of the resistant line is shifted to the left and becomes more sensitive to doxorubicin (Figure 28). The LD_{50} is more indicative of the parental cell line, thereby indicating that sensitivity is restored to the resistant line. To confirm that the shift of the LD_{50} seen in the resistant line was valid due to the inhibition of COX-2 and not due to toxicity of indomethacin, MDA-MB-231 was exposed to the same concentrations of indomethacin only and to indomethacin plus the LD50 of doxorubicin (rounded to 0.01 μM) to ensure no cell death or shifts in the lethal dose when administered with doxorubicin (Figure 29). Also, SD231RD2 was exposed again to indomethacin this time using two added controls, a treatment with indomethacin (50 μM)

only and another using only the LD_{50} (using a rounded value of 0.05 μM) of doxorubicin. The figure clearly demonstrates the shift in the LD_{50} when indomethacin is added (Figure 29).

Furthermore, we also performed an ELISA assay on the wild type and resistant lines for PGE_2, an end product of COX-2. A typical standard curve of PGE_2 is given in Figure 30. The data indicates that the wild type line produced 15.95 pg/mL of PGE_2, however the resistant line showed a much higher abundance of PGE_2 at a concentration of 393.91 pg/mL, thus giving a fold increase of 25 (Table 4).

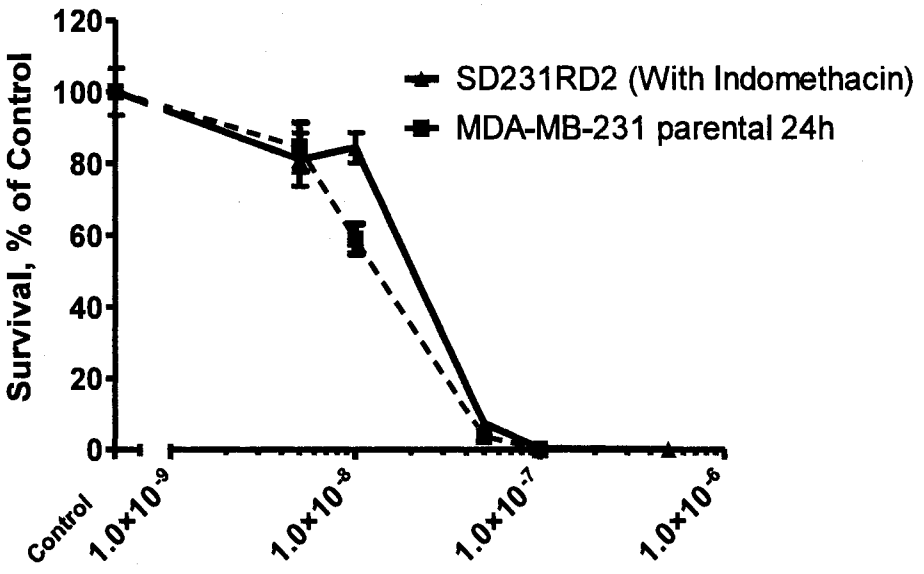

Figure 28. Clonogenic Survival of SD231RD2 with inhibitor Indomethacin (50 μM). Results show that in the presence of 50 μM indomethacin the LD_{50} shifts to the left and resembles the parental cell lines LD_{50} when exposed to doxorubicin (M). Indomethacin and doxorubicin remained on the cells for 24hrs in a combination treatment. Resistance of the cell line diminishes. The figure represents one trial.

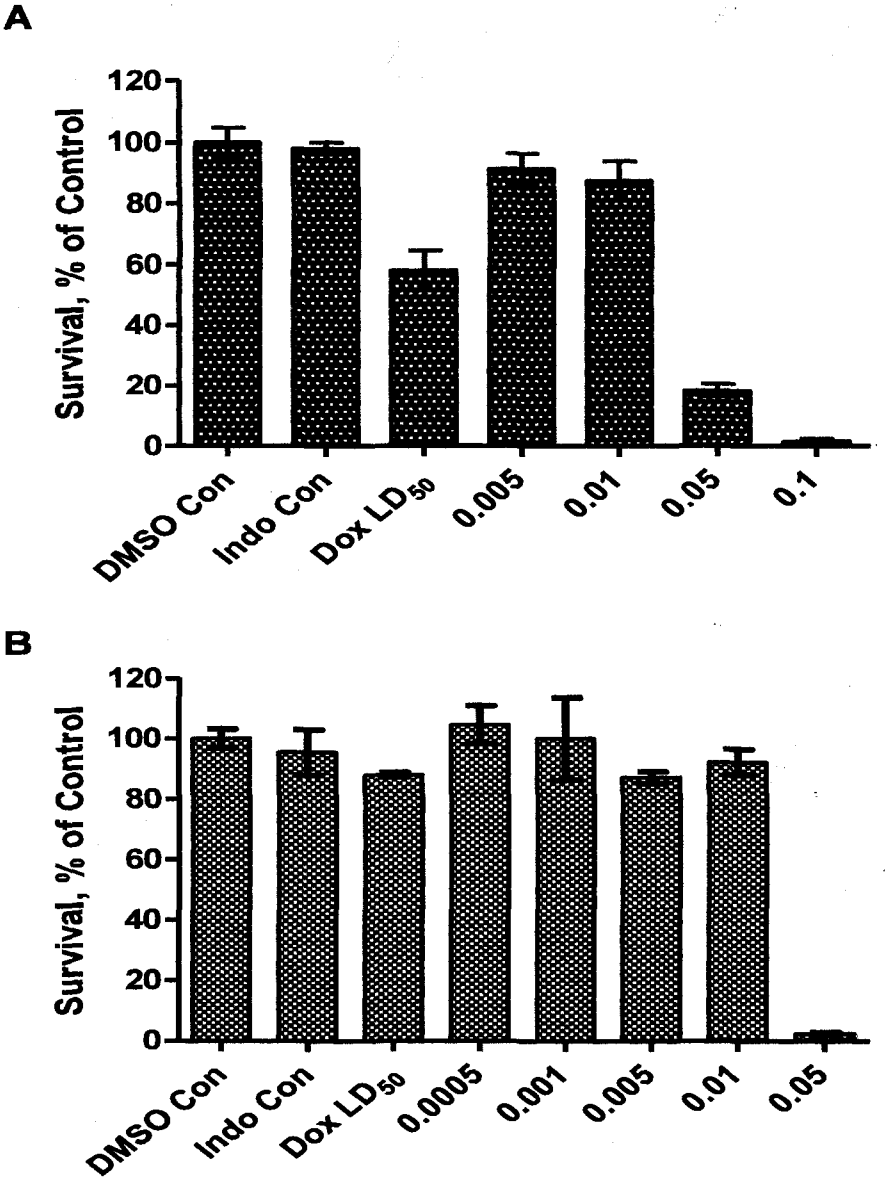

Figure 29. Clonogenic Survival of MDA-MB-231 and SD231RD2 with inhibitor indomethacin (50 μM). Results show that in the presence of 50 μM indomethacin only there is no toxic event occurring when compared to the control for the wild type cell line (B) and the resistant line (A). Also when comparing the LD_{50} values of doxorubicin only to the LD_{50} of doxorubicin (0.01μM) + indomethacin (50 μM) in the wild type line (B) there is no difference within error. However, when comparing the LD_{50} (0.05 μM) of doxorubicin only and then in combination with indomethacin for the resistant line (A) we find a significant increase in cell death. Combinations concentrations of indomethacin and doxorubicin are seen in the bars represented as concentrations in μM.

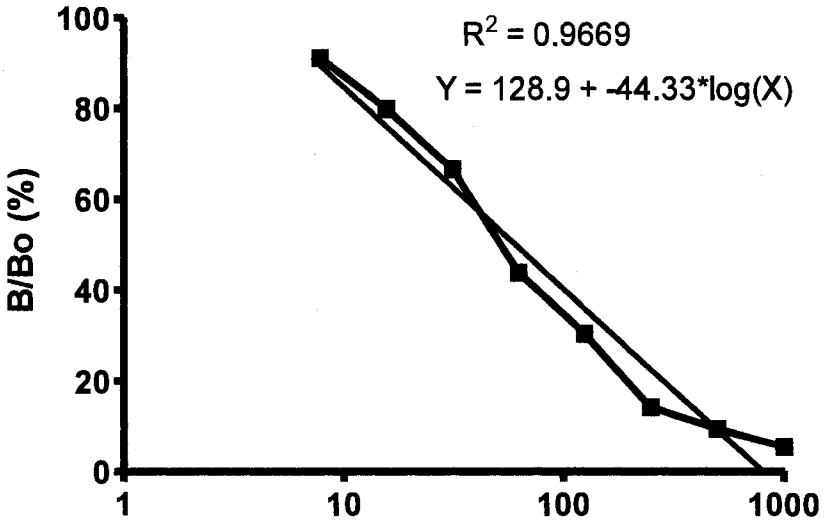

Figure 30. ELISA standard curve for PGE2 where y-axis is B/Bo % and x-axis is concentration in pg/mL. The unknown samples are determined by the log regression line shown on graph.

Treatment	COX-2 (ng/mL)	MMP-3 (ng/mL)	PGE_2 (pg/mL)
MDA-MB-231	0.73	0.35	15.95
SD231RD2	2.18	0.62	393.91

Table 4. Wild type and resistant ELISA data for COX-2, MMP-3 and PGE_2. The table represents only 1 trial of duplicate readings.

In summary, it appears that when doxorubicin is added to the resistant cell lines to promote apoptosis, there is a protection provided by elevated MMP-3 and COX-2. Furthermore COX-2 also stimulates the production of PGE_2, adding more resistance

against cell death. However, the mechanism of this process is still unclear. The wild type cell line has no over expression of gene products or proteins, therefore is not protected and will undergo apoptosis through programmed cell death (Figure 31).

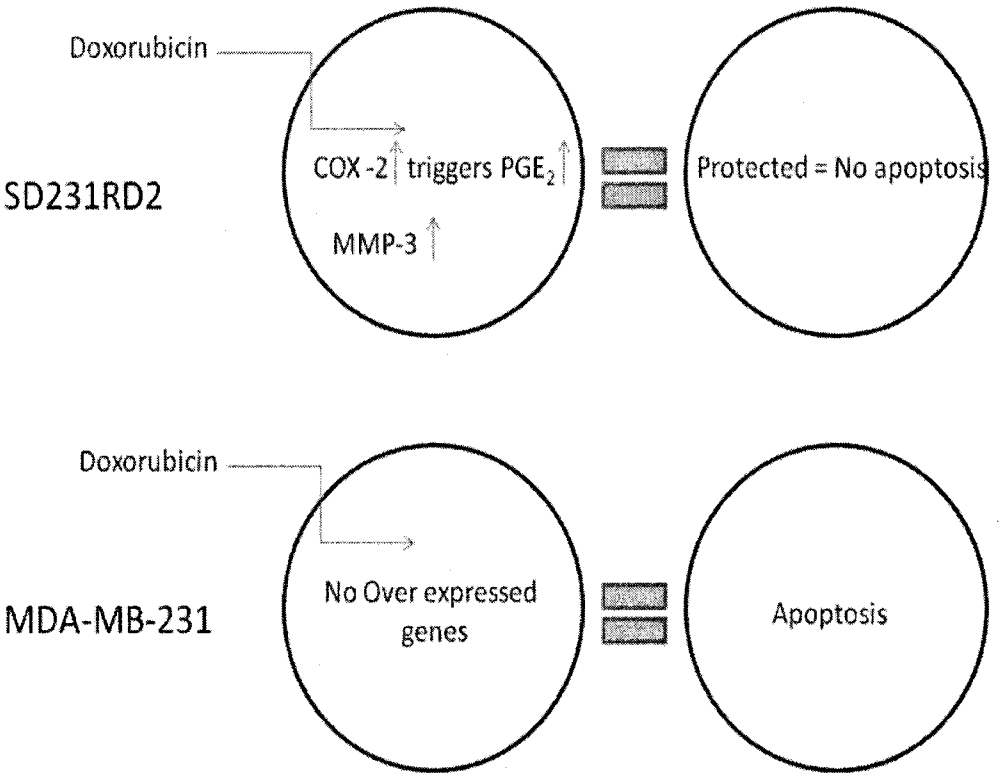

Figure 31. Conditions occuring in resistant cell lines vs wild type.

CHAPTER 5

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion of Results

The main objectives of this study were to determine gene expression profiles and possible biomarkers for treatment of resistant breast cancer cell lines by providing data relevant to the following tasks:

1) Preparation of a resistant breast cancer cell line

Determining the LD_{50} concentrations of the drugs of interest to the wild type cell line is the first step in developing a resistant cell line. This value is needed to determine which concentrations are required to initiate chronic drug exposure to wild type cells. Additionally, the LD_{50} values of wild type lines are needed to compare to resistant lines once developed to confirm resistance. One research group prepared ovarian carcinoma resistant cell lines using an approach similiar to the one described here (22). Other research groups exposed cells to a single dose of 200 nmol/L for 24 h then harvest the remaining cells. To categorize these cells as resistant reseachers performed modified colony formation assays using a drug treatment time of 24 h and cultured for 12 days, which is very similar to the technique used in this study (23). Purchased cell lines that

have target gene knockouts that are known to cause resistance to anticancer agents have also been used.

From the results, parental MCF-7 and MDA-MB-231 breast carcnioma have very similar response to a 1h treatment with cisplatin giving an LD_{50} of 3.13 x 10^{-4}M and 3.33 x 10^{-4}M, respectively (Figure 7). As an initial comparison we choose a known efficacious concentration, the LD_{50}, to determine capacity for resistance. To develop the resistant cell lines we maintained the cells in drug concentrations less than, equal to and higher than the LD_{50}.

However, with respect to doxorubicin, the cell lines behave slighty different in comparison to cisplatin. MCF-7 is a ER^+ carcinoma and was slighty more resistant to doxorubicin, giving a LD_{50} of 6.85 x 10^{-7} M while MDA-MB-231 was more sensitive, giving an LD_{50} of 1.85 x 10^{-7} M (Figure 8). Simlar values are reported in the literature (LD_{50} for doxorubicin treatment in MDA-MB-231 cells reported as 0.25 x 10^{-7}M) (23). Again, the MDA-MB-231 resistant lines were developed with the same method as the MCF-7 resistant lines.

The first technique used to verify resistance was mitochondrial dehydrogenase activity assay. However, the results did not support predicted resistance. The prediction was that resistant MCF-7 carcinoma exposed to 10^{-4}M cisplatin would have the highest degree of resistance, followed by cells exposed to 10^{-6}M then 10^{-7}M cisplatin. This trend was not seen in the data; MCF-7 cells exposed to 10^{-6}M cisplatin had a higher degree of resistance (Figures 9-11) in comparison to cells exposed to 10^{-4}M cisplatin. In alamarBlue assays conducted with the wild type MDA-MB-231 and resistant cell lines, survival was only 10% higher in the resistant line than the wild type at every tested

concentration (Figure 13). To further test the results obtained from the alamarBlue assay, clonogenic survivals were conducted as a measure of resistance.

In the clonogenic survivals using MCF7 wild type and resistant lines, at a concentration of 100 μM cisplatin, there was an increase in survival of 40% in the SDM7-RC7 and SDM7-RC20 lines which are resistant to 10^{-4} and 10^{-7}M cisplatin, respectively. However, there was not a significant shift in the LD_{50} concentrations indicating that there is limited or no resistance to the drug (Figure 12). It was unfortunate that no further testing could be performed with these resistant lines because of their tenuous growth patterns. The unstable growth patterns occured because the vendor used for fetal bovine serum (FBS) was changed from Gibco FBS (Invitrogen) to PAA FBS (PAA laboratories, USA). This change in serum instantly caused decreased cell proliferation and changes in morphology in resistant as well as parental cell lines. Cells were maintained in this serum for three months before returning to the origianl FBS vendor, Gibco. MDA-MB-231 developed into colonies in PAA and were not affected to the same degree as MCF-7 carcinoma, and were successfully adapted from the PAA FBS to the Gibco FBS.

MDA-MB-231 resistant lines were also tested by clonogenic survials. After a 1 h exposure to doxorubicin, there was no difference in the LD_{50} between the resistant and wild type cell lines (Figure 14). The exposure time of drug treatments were increased to 24 h, hoping that an increased exposure would not affect the resistant line but would shift the LD_{50} of the parent line. After a 24h exposure, the resistant line had a 5 fold shift in the LD_{50} towards resistance when compared to wild type (Figure 15). These results are

simliar to data reported in another study in which a shift in the LD_{50} in addition to increased surival at higher concentrations were observed (23).

To test both parental and resistance lines with continous exposure to doxorubicin, a six day viability assay was employed. The results of this assay provided the strongest support for resistance. At day 6 and 0.01 μM doxorubicin, the resistant line had a survival of 80% compared to 20% survival for the parental line under the same conditions (Figure 16). This shows a 60% difference in survival response to continous drug exposure. It is predicted that if the assay was prolonged to 9, 12, and ,15 days then the parental cell line would have 0% survival while the resistant line would continue to survive and proliferate.

While RNA samples were being processed by the Winber Research Institute, further testing was performed to examine the extent of damage to the parental cell line and resistant line on day 6 of the six day assay. A live/dead assay was employed to determine the population of injured, dead and living cells on these cell populations. After six days of continuous treatment, the resistant lines had the same levels of living, injured and dead cells as the parental which had not been exposed to drug. This data indicated that the drug had little effect on the resistant cell line at the given concentration. However, the parental line that was treated for six days with 0.01 μM doxorubicin showed 40% of the cell population being injured and in the first stage of apoptosis (Figure 17). The ability of the resistant cell line to tolerate continuous drug exposure without injury is further supported by cell cycle analysis where we see that wild type cells exposed to treatment undergo a block in G2/M and a 20% increase in cells undergoing apoptosis. Comparatively, the resistant line shows no changes in cell cycle and continued cell cycle progression,

indicating that these cells are not affected by drug exposures (Figure 18). Flourescence microscopy was employed to determine whether cell death is occuring by the apoptotic or nercrotic pathway. According to the results, the wild type cell line dies by the apopotic pathway when exposed to a six day continuous treatment of doxorubicin. However, the resistant cell line experienced little cell death but also had a slightly higher percentage of cells in the apoptotic pathway (Table 2). The images in Figure 18 demostrate the parental morphology changes which is consistent with reduced proliferation. It is interesting that there is no morophology changes in the resistant line compared to the parental. The only change is the rate of proliferation which is slightly lower when compared to wild type.

1. Determine by microarray analysis which genes are overexpressed in the resistant cancer cell line compared to wild type.

Microarray analysis gave us a total of 712 differently expressed proteins. We decided to focus on genes in the resistant population which were over expressed by two fold or higher in comparison to the wild type. We identified 528 genes that were over expressed at this level. We isolated the top ten genes that were overexpressed (Table 3) and of these ten genes we chose two targets to investigate further. These genes were COX-2 and MMP-3 which had fold differences of 11.96 and 14.93, respectively.

COX-2 is part of arachidonic acid metabolism which occurs in the immune system and the brain. Inflammation increases COX-2 expression which results in the enhanced production of PGE_2 (24). Prostaglandin synthesis begins with phospholipase (PLA2) mediated release of arachidonic acid. COX-2 converts arachidonic acid into PGH_2, which is then metabolized to many Prostaglandins (PGs), including PGE_2 (Figure 6). COX-2 has

variable expression in comparison to COX-1 and is highly expressed in cells experiencing stress whereas COX-1 is constitutively expressed. These different cyclooxygenases also have different biological functions in the cell (25). COX-2 is a protein of interest given that induced levels of COX-2 induce genomic instability in a normal breast line (MCF10A) and genomic instability is one of the hallmarks of cancer. Three downstream targets of COX-2 activity that have a role in metastasis are found to be urokinase plasminogen activator, interleukin 8 (IL-8), and interleukin 11 (IL-11). COX-2 mediates these events through the PI3-kinase/Akt signalling pathway. It is also found that COX-2 induces expression of the anti-apoptotic protein Bcl2, which is known for causing drug resistance in many carcinomas to a variety of anticancer agents, thus providing a rationale for doxorubicin resistance in the cancer initiating phenotype (26).

Matrix metalloproteinases, MMP-3 (stromelysin) degrades several extracellular matrix molecules (ECM), such as cartilage proteoglycan and type II collagen. It is known that interleukin-1β, a proinflammatory cytokine, induces expression of MMP-3 and prostaglandins (27). MMP-3 over expression in both MCF-7 and MDA-MB-231 cells leads to increased cell numbers and tumor volumes. The presence of MMPs in tumors can lead to complex treatment challenges as these proteases are able to promote or impede tumor relevant processes such as proliferation, apoptosis, angiogenesis and metastasis (28). The mechanism for MMP-3 induction and subsequent increased resistance is still unclear. Although microarray results indicated over expression of RNA, protein over expression was not detected in Western blot (Figure 20-22) or ELISA assays (Table 4).

2. Identify the pathway or mechanism in which these genes of interest are involved;

One reason for identifying differential gene expression in resistant cells is to identify mechanisms and cellular functions that mediate resistance. Since the genes COX-2 or MMP-3 were not indentified in Western blot analysis (Figures 20-22), ELISA assays, a more sensitive technique were used. However, the ELISA results are similar to Western blot analyses, as there was no COX-2 or MMP-3 expression in the wild type carcinoma. The limit of the detection of the COX-2 ELISA kit was 2.15 ng/mL, while the limit of detection for the MMP-3 ELISA was 0.62 ng/mL. From Table 4, the concentrations of COX-2 and MMP-3 that are presented for the parental line are negligible because the values are below the detectable limits of these assays. The resistant line concentrations of these genes are just above the level of detection of these assays; therefore these values are not reliable and cannot conclude any relevance.

To test for COX-2 up-regulation, the measurement of eicosanoid production can demonstrate whether the protein is actively inducing product formation. PGE_2 was tested due to its putative role in causing resistance. PGE_2 is known to stimulate angiogenesis, invasiveness and inhibit immune surveillance (28). ELISA assays were performed to determine if over expression of COX-2 would cause an increased level of PGE_2. The results indicated a 25 fold increase in the production of PGE_2 when compared to the wild type cell line (Table 4).

To further investigate the mechanisms in which the process of up regulation of PGE_2 is caused in the resistant cancer cell line, SD231RD2, microarray data was examined for genes that are related to the arachidonic acid metabolism. The PLA2 gene is responsible for regulation of the enzyme phospholipase and therefore stimulation in the production

arachidonic acid. An increase in this gene could account for the increase in PGE_2 that is shown in the resistant line (because of simple metabolism an increase in an enzyme requires synthesis of more reactant, therefore product build-up). However, the microarray data shows a decrease in the PLA2 gene in the resistant line with a fold difference of 0.45 when compared to the parental cell line. Also COX-1 was not reported in the microarray analysis of being over or under expressed. This proposes that it is solely the action of increased levels of COX-2 that is stimulating the production of PGE_2.

COX-2 expression is an important factor for angiogenesis. Angiogenesis is mediated by migration and proliferation of the host endothelial cells and is a required for tumor growth. Vascular endothelial growth factors (VEGF), transforming growth factor (TGF), basic fibroblast growth factors (FGF) and chemokines (IL-8) are implicated in angiogenesis in lung cancer (29). Microarray data for SD231RD2 indicates that the genes VEGF, TGF are present and are overexpressed in this study by 2.3 fold, while FGF is increased by 4.2 fold. Thus, indicating that the cells are capable of performing angiogenesis. Also it has been shown by Patel and Chiplunkar that in lung cancer PGE_2 can also increase cyclin D1 and VEGF through Wnt signalling. This can also contribute to the increase seen in VEGF. Epidermal growth factor receptor (EGFR) also needs to be increased for the production of tumors, and is seen to have a 2.5 fold increase in the microarray data of SD231RD2.

Extracellular matrix proteins (ECM) are important in tumors for metastasis. MMP-3 was shown to be over expressed in the microarray data of SD231RD2 resistant line. COX-2 was demonstrated to up-regulated ECM genes such as MMP-3 and laminin 5,

proteins involved in migration and invasion (29). The data indicates that laminin 5 is a subunit of the gene LAMA3 which is overexpressed 4.4 fold in this study.

MDR-1 was also found in this study to be increased by a 2.2 fold difference when compared to the wild type cell line. Drug resistance often involves MDR-1 which encodes a transmembrane glycoprotein p-170 (P-gp). This protein is responsible for protecting cancer cells by pumping out cytotoxic agents, such as doxorubicin out of the cytoplasm and making chemotherapy ineffective (30).

4. Determine if an inhibitor of these genes will restore sensitivity to the anticancer agent in the resistant line.

Targeted inhibitions of COX-2 and PGE_2 are regarded as potential strategies to prevent the presence or progress of cancers. However, there is risk associated with the inhibition of COX-2. Development of cardiovascular disease has been associated with using the inhibitor of COX-2, celecoxib, (originally developed for arthritis and pain) for the treatment of lung cancer, but it is still being used since it has been shown to induce apoptosis in lung cancer (29). Celecoxib has also been shown in MDA-MB-231 cells to enhance the intracellular accumulation of doxorubicin, possibly via modulation of NF-kB activity (30). Ciglitazone is an inhibitor of COX-2 and has been used in a variety of carcinoma trials (31). Other inhibitors include: nonsteroidal anti-inflammatory agents (NSAIDS) and indomethacin, a non-specific COX inhibitor. Doses of 100 to 150 mg per day of indomethacin are used to treat rheumatoid arthritis for a period of 4-6 weeks. The comparable concentrations used *in- vitro* are 50-300 μM (32). Since indomethacin is known to reduce invasion and metastasis in breast carcinoma, indomethacin was used to

inhibit COX-2 activity in our resistant cancer cell line. Upon treatment with indomethacin, the resistant line experienced increased sensitivity to doxorubicin, giving an LD_{50} concentration of 2.04 x 10^{-8}M for doxorubicin. This value is similar to the parental line with an LD_{50} of 1.16 x 10^{-8} M for doxorubicin (Figure 28). This indicates that inhibited COX-2 activity through the use of an inhibitor may reverse the resistance and restore sensitivity which is comparable to the parent line. It has also been shown that when indomethacin is applied to the parental cell line no toxic event was observed. The wild type cell line behaved the same in both the presence and absence of indomethacin, while the resistant cell line was sensitivity was regained (Figure 29). Supporting studies used a MCF-7 cell line that is resistant to low doses of doxorubicin. They indicated that a selective inhibitor for COX-2 called NS-398 restores the resistant lines sensitivity by increasing the intracellular doxorubicin accumulation in the cell, thereby inhibiting P-gp expression (30).

5.2 Conclusions and Recommendations for Further Study

Several problems were encountered during the course of this study. The development of a resistant cell line can take from four months up to one year. Also, the first line developed was the MCF-7 cell line which was resistant to cisplatin. However, due to a change in FBS serum, the cell line ceased to proliferate, which resulted in the inability to fully characterize this cell line. Therefore, there is only a small amount of data which supports drug resistance with this cell line. Ultimately, the MCF-7 resistant lines could not be studied due to the loss of all of these lines. MDA-MB-231 resistant lines were developed late in the research progress, which limited the amount of secondary testing

that could be performed. Testing to determine resistance took longer than anticpated and microarray results took six weeks to analyze which further limited the amount of time for additional study.

Microarray analysis show over expressed RNA species, but when we tried to validate this data by experiments which probed for protein product, a corelation was not observed. A possible explanation could be that the overexpressed mRNA from the microarray results may be degraded prior to translation due to a short poly A tail or alternative mechanisms such as ubiquitination which inhibit the mRNA being translated into protein. To confirm the microarray analysis semi quantitive RT-PCR was used. The PCR results demostrated similar results to the microarray data, indicated that mRNA of the gene products are present. The bands seen for the amplification of the gene products show the over expression observed in microarray studies (Figure 23-24). The resistant lines are producing increased levels of mRNA for these proteins when compared to wild type. To determine if gene expression was changing over time, we isolated fresh RNA and ran SQ-PCR and obtained similar results (Figure 25). These results ultimately lead to questioning the validity of RNA microarray and supporting the use of protein array analyses to determine biomarkers for resistance. Future studies in our lab include protein arrays on resistant lines and comparing that to both wild type protein array and the microarray data. Other research groups conclude that protein arrays would be the superior approach since they more closely resemble biochemical activity and given that protein concentrations do not correlate to mRNA levels (23,33).

Further testing of the MMP-3 mechanism and using inhibitors for this protein would distinguish its mode of action. TIMP (tissue inhibitor of metalloproteinase) is an

inhibitor of MMPs. There are different TIMPs for the different 25 MMPs that are currently known (27). Inhibiting the action of MMP-3 would allow us to determine if this protein is another contributing factor to resistance that is observed with doxorubicin.

Determining the mechanism by which both of these proteins are contributing to resistance is essential. Finding precursors to COX-2 and MMP-3 and determining if these proteins are involved in the same mechanism is crucial for determining factors of induciblity of these proteins. An initial target of this study should be IL-1β. Silencing this cytokine and then measuring the levels of MMP-3 and COX-2 products downstream will confirm if IL-1β induces these proteins (27).

In conclusion, it appears that drug resistance in cancer cell lines can be reversed and drug sensitivity restored. Selective inhibitors of COX-2 and other specific genes (known for resistance) are in need to prevent further damage to surrounding cells and limiting side effects such as cardiovascular problems. Also, it appears that microarray analysis does not corelate with protein data. Protein expressions of over expressed genes were not observed, however end products of the pathways were increased as demonstrated with COX-2 and PGE_2. Therefore, protein arrays should be used for future studies. Finding a mechanism by which COX-2 becomes induced in cancer cells and how PGE_2 contributes to drug resistance and its overall affects on the cell should be further studied by determining how it there pathways induce tumor survival.

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