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DNA Damage induces Invasion of MCF-7(Human Breast Adenocarcinoma) Cells

A thesis Presented

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Abstract of the Thesis

DNA Damage induces invasion of MCF-7 (Human Breast Adenocarcinoma) Cells
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Breast Cancer is a common malignancy in American and European women, and approximately one-third of these women develop metastasis and die from the disease. Chemotherapy is widely used to treat breast cancer and is known to extend survival of patients. Despite this fact, a small percentage of cells can endure and gain resistance to these treatments. Clinicians have sought to increase the time and intensity of these doses to reduce resistance whereas others have lowered the doses to reduce toxicity. The chemotherapeutic agent Doxorubicin is used as an adjuvant treatment for tumors of the lung and breast. Its anti-tumor activity is thought to occur through DNA breaks in the tumor cell. Our findings indicate that low dose doxorubicin induces SRC and Fyn tyrosine kinases and the invasiveness of MCF-7(Human breast Adenocarcinoma, ER+ luminal) cells. Using the MCF-7 cell line, we explored the effects of various Doxorubicin doses on the expression of SRC and Fyn pro-invasive genes identified from our RNA sequencing data. Our results suggest that the *in vitro* sub-lethal Doxorubicin dose falls in the concentration range of 400 nM to 600 nM, and it is mainly inducing Fyn at the transcriptional level. Our subsequent experiments focused on Fyn mRNA up-regulation in breast cancer cells, since previous studies have shown Fyn to play a role in progression and metastasis in malignancies such as prostate cancer. Interestingly, knockdown of p53 via siRNA, suppressed this induction, indicative that Fyn upregulation is occurring as a response to DNA damage. To further complete our study, we determined if SRC and Fyn up-regulation is a universal mechanism associated with other DNA Damaging agents and chemotherapeutics. Our results indicate that MCF-7 cells treated with UV light, etoposide, Camptothecin and Taxol do not show a significant upregulation of SRC and Fyn genes, thereby confirming that induction of these tyrosine kinases is Doxorubicin specific. Lastly, we determined Doxorubicin is not inducing Fyn mRNA through an Epithelial-Mesenchymal transition but rather as a Doxorubicin specific DNA damage response.

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List of Abbreviations

MCF-7 cells	Michigan Cancer Foundation-7 Cells
DOX	Doxorubicin
nM	Nano molar
μM	Micro molar
EMT	Epithelial to Mesenchymal transition
2-D	Two-Dimensional
3-D	Three-Dimensional
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
RPMI	Roswell Park Memorial Institute medium
UV	Ultra-Violet
PARP	poly(ADP-ribosyl) transferase
Chk1	Checkpoint Kinase 1
SDS-PAGE	Sodium Dodecyl sulfate polyacrylamide gel electrophoresis
SiRNA	Small interfering RNA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
kDA	Kilo Dalton
FBS	Fetal Bovine Serum
MNE	Mean-Normalized Expression
AS	All-Star Negative Control
Veh	Vehicle Control
RFU	Relative Fluorescence Units
mJ	Mille-Joules
mRNA	Messenger RNA

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I would like to first thank Dr. Hannun for allowing me to conduct research in his laboratory. His support, encouragement, and patience has been influential and essential to my understanding and completion of the project.

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Lastly, I would like to thank all the members of the Hannun and Obeid lab for all their encouragement and support.

Introduction

Breast cancer is a common malignancy among women in the United States, this occurs when cells begin to grow abnormally and form mass of cells in the breast. Respectively, these mass of cells can lead to the formation of a malignant tumor. Breast Cancer can be divided into four groups, based on their molecular features. The first two types are Luminal A and B, which are estrogen receptor positive, luminal A subtypes typically have a better prognosis then luminal B. The third type is HER 2 positive, which typically grow more quickly than other breast cancer cell types. Lastly, breast cancers of basal origin are called triple-negative since they lack estrogen and progesterone receptors. These four types of breast cancer, have individualized treatments, but they are all usually accompanied by adjuvant chemotherapy which has helped improve prognosis and survival.

In addition to chemotherapy, other forms of therapy may include hormone therapy and targeted therapy depending on the breast cancer type and severity. In our study we used the MCF-7 cell line, which was derived from a patient with metastatic breast cancer in the 1970's [1]. MCF-7 cells are classified as Luminal A and estrogen receptor positive cells, they retain several characteristics of differentiated mammary epithelium, including their ability to form dome-like structures in 2D monolayer culture [2]. These characteristics of MCF-7 cells have allowed them to be a great *in vitro* model for Human breast adenocarcinoma. In our study, we focused on the chemotherapeutic Doxorubicin, which is clinically used for the treatment of many different types of tumors [2], and is often used in adjuvant chemotherapy treatments. Clinical trials have found that these types of treatment increase the overall survival of women diagnosed with breast cancer [3].

Other Chemotherapeutics and DNA damage agents such as Etoposide, Camptothecin and Taxol are used in the treatment of breast and other human cancers. Etoposide, a topoisomerase 2 inhibiting anticancer drug has been widely used for the treatment of Lymphomas, testicular, lung and ovarian cancer [19]. Camptothecin, a compound that has been demonstrated to be effective against a broad spectrum of tumors, works by inhibiting DNA topoisomerase 1 which is essential for cellular metabolism and survival [23]. Pacltaxel (Taxol), an anti-cancer agent that interferes with the growth of cancers of the breast, lungs and ovaries and is also used in conjunction with other chemotherapeutics.

Studies have sought to established whether the dose and intensity of chemotherapy treatment has a significant effect on the relapse rate. In addition, some studies have established that the dose of chemotherapy should not be reduced if maximum survival is to be achieved [3]. Although increasing the dose of these chemotherapeutics kills cancer cells, there is a great deal of systemic toxicity and side effects that these patients must endure. Physicians have sought to reduce the problem by decreasing the dose and dose intensity, but as expected the number of patients that have tumor recurrence greatly increases [3]. In a study that was done by the Cancer and Leukemia Group B(CALGB), they conducted a randomized trial of different levels of doses and dose intensity of chemotherapeutics in women with stage 2 estrogen-receptor positive breast cancer. The women were divided in three groups, one group receive a combination of Cyclophosphamide, Doxorubicin, and fluorouracil, another group received the same dose but in a shorter time-period thus increasing the intensity of the dose. Lastly the third group received half the total dose and half the intensity as the first two groups. In a 3 year follow up, the two groups treated with high dose chemotherapeutics had a longer disease-free survival then the third group. The third group of women showed a higher percentage of local chest wall recurrence, in addition some of these women had recurrence in the site of the primary(original) tumor [3]. The physicians that conducted this study, attribute these results to a dose response effect in which the effect of the chemotherapeutics increases proportionally with increasing doses, and dose intensity.

A critical aspect of the study above is that the group that receive the low dose doxorubicin treatment, had recurrence of disease in different body sites, which suggest that in addition to site-specific cancer recurrence, this low dose doxorubicin treatment induced invasion and metastasis to different body sites [3]. The mechanism by which this occurs has yet to be elucidated and it is what we seek to explore and understand in our study using MCF-7 breast cancer cells. RNA Sequencing data from our laboratory [Figure 1.10] shows up-regulation of SRC family tyrosine kinases after low dose doxorubicin treatment. SRC family tyrosine kinases are commonly overexpressed in a variety of epithelial and non-epithelial cancers of the breast and colon, in addition they have been showed to be involved biological phenomenon's such as tumor progression, migration, invasiveness and resistance to apoptosis [15]. In our study, we investigate whether these kinases are activated through induction of specific DNA damage pathways, using the clinically relevant chemotherapeutic Doxorubicin whose anti-tumor activity occurs by inducing DNA double- strand breaks in tumor cells.

In our study, we found that Fyn mRNA is the major tyrosine kinase that is up-regulated following sub-lethal Doxorubicin. Fyn is generally associated with neuronal and T-Cell signaling under normal cellular circumstances, but in cancer, it's kinase activity is often dysregulated. Studies have shown that overexpression of Fyn can lead to dysfunctional cellular motility and an increase in anchorage-independent growth [11]. Interestingly, studies have also reported Fyn to play a role in Epithelial to mesenchymal transition which is a biological process that occurs when epithelial cells lose their cell polarity and adhesion to become mesenchymal-like cells. Cancer cells that have undergone an EMT, tend to more invasiveness and gain migratory ability. In addition, there are number of molecular markers that are involved, genes may be turned on such as Vimentin, snail, and twist and genes. Our data suggest that up-regulation of Fyn in MCF-7 breast cancer cells does not result in an EMT (Epithelial to mesenchymal transition) [Figure 4.2a], rather the precise signaling pathway that Fyn is inducing remains of great interest and it is what we seek to explore in our study.

It has been widely accepted that events such as DNA damage from double- strand breaks and chemical damage to DNA, result in an increase in p53 levels that is proportional to the extent of DNA damage. Our data demonstrates that Fyn is the major downstream target of p53, since their up-regulation is diminished in the absence of p53[Figure 4.1c]. Moreover, Chk1 which is an effector kinase of the DNA damage response, was not shown to take part in the up-regulation of SRC and Fyn Tyrosine kinases [Figure 4.1c]. Taken together, our data suggest that p53 is necessary to give rise to the up-regulation of the pro-invasion gene Fyn, after treatment with low dose doxorubicin.

Many studies have reported that inhibition of SRC kinase activity, inhibits cell growth and migration in both *in vitro* and *in vivo* systems [13]. Dasatinib a SRC family kinase inhibitor, is currently used in the treatment of imatinib-resistant leukemia's and currently is in phase 2 clinical trials for the treatment of solid tumors. It has been reported that when Dasatinib is paired with Doxorubicin they synergistically decrease proliferation and viability in MCF-7 cells [13]. We explored these reports in our study and our data indicates that multi-drug treatment of Dasatinib and Doxorubicin synergistically decreases SRC gene expression (Figure 5.3a). Collectively, these results can provide a rationale for including Dasatinib in multi-drug regimens to suppress invasion of primary breast cancers tumors [13].

Materials and Methods

2.1 Cell lines, Cell Culture, and Chemotherapeutics

MCF-7 breast carcinoma cells were obtained from ATCC (American Type Culture Collection).MCF7 cells were grown at 37°C with 5% CO_2 in 10% FBS in RPMI culture medium(InVitrogen). Cells were sub-cultured in 60mm dishes (250,000 cells) and the medium was changed 1 hour before the start of experiments. Doxorubicin, Camptothecin, Etoposide and Taxol were all obtained Sigma (St. Louis,MO,USA), aliquots and dilutions were made accordingly to experiments.

2.2 Protein Extraction and Immunoblotting

To extract cellular protein, the cells were harvested in ice-cold RIPA buffer and lysed by sonication. Protein concentrations were estimated by the Bradford assay, and aliquots of lysates were mixed with equal volumes of 4X laemmli(Bio-Rad) buffer, vortexed, and boiled for 15 minutes. Protein samples were separated by SDS-PAGE and analyzed via western blot.

2.3 List of primary antibodies

All primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA) and are listed below. Dilutions were made according to Manufactures protocol.

Name	Manufacturer Number	Dilution	Source
Total SRC	#2123	1:1000	Rabbit
FYN	#4023	1:1000	Rabbit
PARP	#9532	1:1000	Rabbit
P53	#9282	1:1000	Rabbit
Actin	#3700	1:5000	Mouse
GAPDH	#2118	1:1000	Rabbit
Total Chk1	#2360	1:1000	Mouse
E-Cadherin	# 3195S	1:1000	Rabbit
Vimentin	#5741	1:1000	Rabbit

2.4 List of Secondary Antibodies

All secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Ca, USA) and are listed below. Dilutions were made according to Manufacturers protocol.

Product Name	Catalog Number	Dilution
goat anti-mouse IgG-HRP	sc-2005	1:5000
goat anti-rabbit IgG-HRP	sc-2004	1:5000

2.5 Real-time PCR

RNA extraction was performed using the Purelink RNA kit from Life Technologies according to the manufacturer's protocol, RNA quality and concentration were verified by Nano drop, after which 1μg or 500ng(depending on the concentration of RNA) was transformed using the Quanta cDNA kit(Gaithersburg, MD, USA) according to the manufacturer's protocol. For qRT-PCR, reactions were run in triplicates in a 96 well plate with each reaction containing 10μl of 2 x iTAQ mastermix, 5μl of cDNA, 1 μl of Taqman primer probe and 4 μl of water. Primer probes were purchased from life technologies and amplified with SRC, FYN, and ACTIN.

2.6 Cell Culture and Reverse transfection siRNA

For siRNA experiments cells were plated in 60mm dishes (250,000 cells) and 24 hours later, they were transfected with 10nM `negative control or 10nM of the desired siRNA using lipofectamine RNAiMax reagent (Life technologies) according to the manufacturer's protocol. After 24hr, cells were incubated in the fresh medium for 1 hr before treatment with Doxorubicin.

2.7 3D Culture with various Dox Doses

For 3-D Culture experiments, MCF-7 cells were grown in Matrigel to simulate a cell's extracellular environment. Matrigel was composed of laminin, collagen 4, nidogen and proteoglycans which resemble the basement membrane of cells. Culture Media contained growth factors and matrigel which allowed MCF-7 cells to form Acni structures in 3D.

Results

Section 1: Sub-lethal Doxorubicin induces SRC and FYN mRNA.

Our initial RNA sequencing data [Figure 1a] with nine members of SRC family tyrosine kinases, show that MCF-7 Breast Cancer cells that are treated with 600nM Doxorubicin are specifically inducing SRC and Fyn mRNA 2-4 fold in comparison to other SRC Kinase family members. Subsequent experiments were performed to validate this up regulation of SRC and FYN tyrosine kinases in MCF-7 breast cancer cells.

SRC Family Kinases	Ensembl Number	AS Vehicle	AS 600nM Doxorubicin	Fold Change
Blk	ENSG00000197122	625	1378	2.2048
Fgr	ENSG00000000938	0	0	0
Fyn	ENSG00000010810	46	171	**3.7173**
Hck	ENSG00000101336	0	0	0
Lck	ENSG00000182866	0	0	0
Lyn	ENSG00000254087	0	0	0
Src	ENSG00000197122	625	1378	**2.2048**
Yes	ENSG00000176105	0	0	0
Frk	ENSG00000111816	0	0	0

Figure 1.1. RNA Sequencing Data.

a.) RNA sequencing was done on the transcriptome of MCF-7 Breast Cancer cells treated with +/- 600nM Doxorubicin., The Gene Ensembl number and Genecards (Human Gene Database) were used to identify the different transcripts from our RNA Sequencing data. Microsoft excel was used to determine the fold change in mRNA expression of cells treated with -/+ 600nM Dox compared to Vehicle control [5].
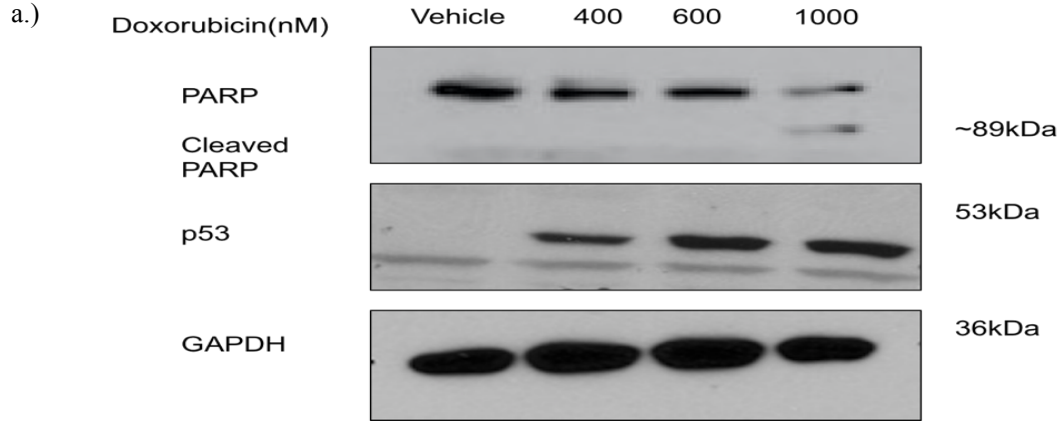
a.)

Figure 1.2. Doxorubicin Dose effect on PARP and p53.

a.) MCF-7 cells were seeded in 60 mm dishes, and cells were stimulated either with Vehicle(DMSO) or Doxorubicin. Twenty-four hours after treatment, cells were collected and immunoblotted for PARP, p53, and GAPDH.

In order to confirm that the doses of Doxorubicin required to induce DNA damage are sub-lethal, we probed various Doxorubicin doses for PARP (poly(ADP-ribosyl) transferase) and p53 protein, PARP is specifically cleaved into 85 kDa polypeptides during apoptosis and p53 is induced as a result of DNA Damage. PARP cleavage was not observed with 400-600nM Doxorubicin treated cells confirming that 400-600nM dose range is sub-lethal. Simultaneously, p53 protein levels increased following treatment with 400nM Doxorubicin confirming the 400-600nM dose range is inducing a DNA Damage response [Figure 1.2a].
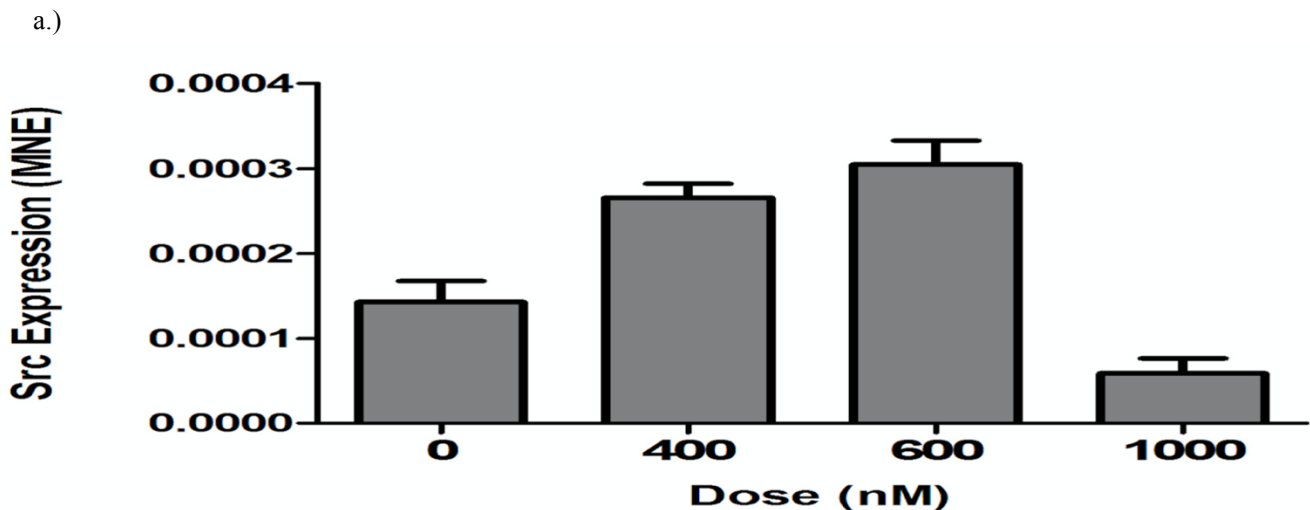
a.)

Figure 1.3. Doxorubicin Dose effect on SRC mRNA.

a.) MCF-7 cells were lysed 24 h after treatment with Doxorubicin. RNA was isolated and transformed to cDNA to be quantified by real-time PCR (qRT-PCR) using primers for SRC and Actin.

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It was important to validate whether sub-lethal Doxorubicin is inducing SRC and Fyn mRNA since our previous RNA sequencing data showed a 2-3-fold induction for SRC and Fyn mRNA. We initially look at SRC mRNA levels following 24-hour treatment with Doxorubicin, using quantitative RT-PCR, in which the expression levels of target gene of interest were normalized to the expression level of Actin reference gene. Our results show a 2-fold mRNA induction following 400nM Doxorubicin treatment and approximately a 3-fold peak mRNA induction at 600nM Doxorubicin treatment [Figure 1.3a].
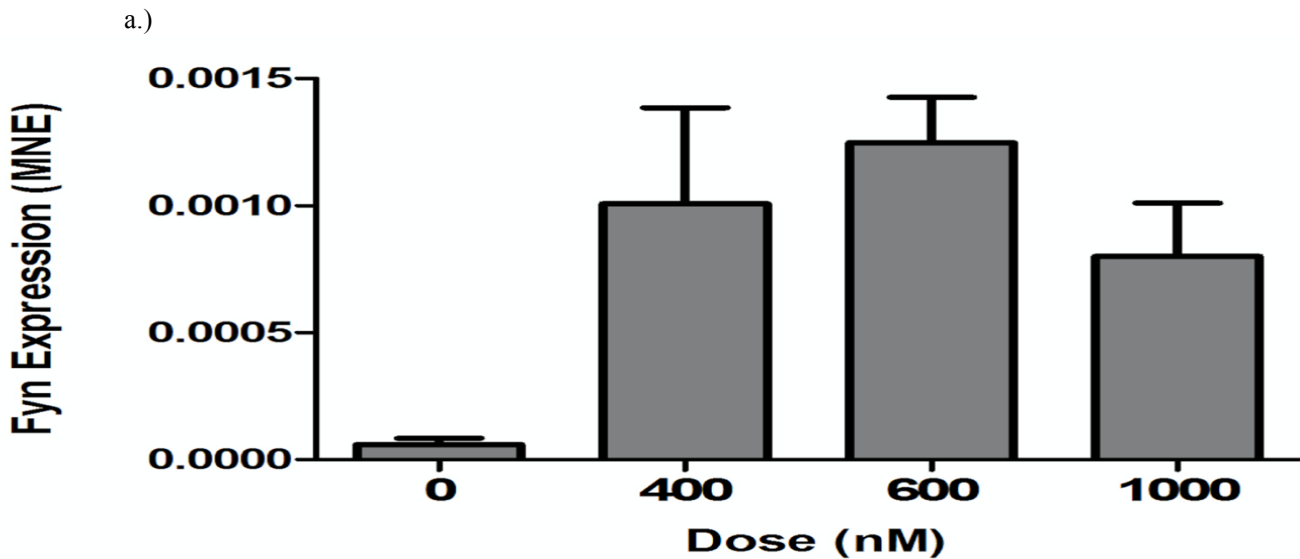
a.)

Figure 1.4. Doxorubicin Dose effect on Fyn mRNA.

a.) MCF-7 cells were lysed 24 h after treatment with Doxorubicin. RNA was isolated and transformed to cDNA to be quantified by quantitative real-time PCR (qRT-PCR) using primers for Fyn and Actin.

Successively, we investigated Fyn mRNA levels following 24- hour treatment with Doxorubicin, using quantitative RT-PCR (as described previously for SRC). Our results indicate that Fyn mRNA levels [Figure 1.4a.] increased 17-fold following 400nM Dox treatment and approximately 21-fold peak mRNA induction at 600nM, far more than SRC mRNA levels, implicating that Fyn is the major transcriptional target following Doxorubicin treatment and may be the main contributor to the pro-invasive phenotype describe previously [3].
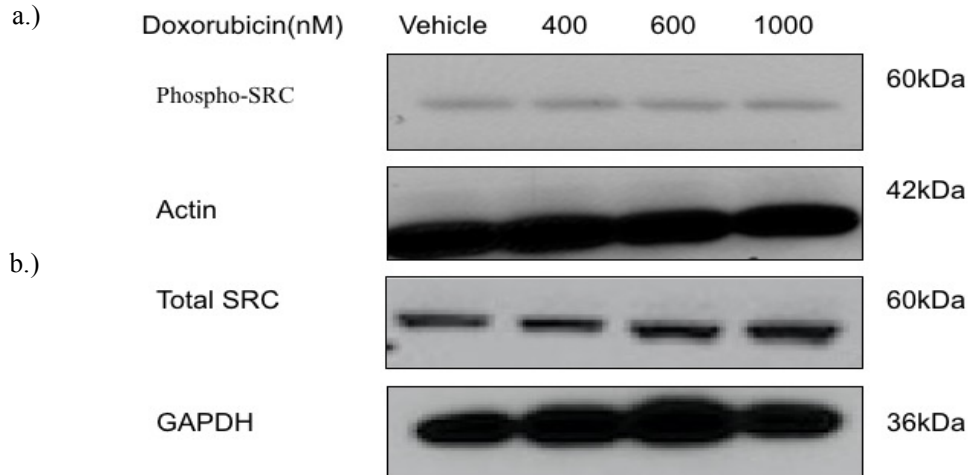
Figure 1.5. **Doxorubicin Dose effect on SRC protein.**

    a.) MCF-7 cells were plated in 60 mm dishes and treated for 24 h with either DMSO(Vehicle)or Doxorubicin, twenty-four hours later after treatment, cells were collected and immunoblotted for p-SRC and Actin.

    b.) MCF-7 cells were plated in 60 mm dishes and treated for 24 h with either DMSO(Vehicle) or Doxorubicin, twenty-four hours later after treatment, cells were collected and immunoblotted for Total SRC and GAPDH.
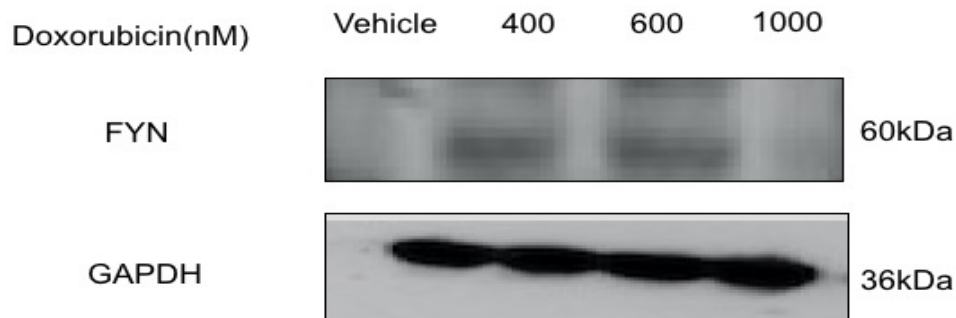

Figure 1.6. Doxorubicin Dose effect on Fyn protein.

    a.) MCF-7 cells were plated in 60 mm dishes and treated for 24 h with either DMSO(Vehicle) or Doxorubicin, twenty-four hours later after treatment, cells were collected and immunoblotted for FYN and GAPDH.

Subsequently, we sought to determine if up-regulation of SRC and Fyn is also occurring at the translational level. According to our results there is no significant induction of phospho-SRC and some increase in Total Src protein levels [Figure 1.5a, b]. In contrast, Fyn protein induction was seen at the 400 and 600nM dose range [Figure 1.6a], with no further induction seen at the 1000nM dose range. Collectively, these results suggest that Doxorubicin is inducing SRC and Fyn protein, in addition to transcriptional up-regulation of SRC and Fyn mRNA.

Section 2: Fyn induction by sub-lethal Doxorubicin is acute and transient with prolonged Doxorubicin.

Once we determined that Fyn is the main SRC family tyrosine kinase that is up-regulated following Doxorubicin treatment, we performed time course experiments in order to determine how long this induction of Fyn is sustained [Figure 2.2a], and if this induction is sustained following Doxorubicin removal [Figure 2.3b.]. Initially, we looked at 24, 48 and 72-hour sub-lethal Dox treated cells and their effect on PARP and p53 [Figure 2.1a]. Our results show that PARP protein is cleaved 48 hours after sub-lethal Dox treatment indicating that these cells are undergoing apoptosis, successively, 72-hour sub-lethal Dox treated cells had no detectable PARP protein[Figure2.1a], indicative of cell death. Simultaneously, p53 protein induction remained constant for 24 and 48 hours following sub-lethal Dox treatment, with a vast decrease 72 hours after treatment [Figure 2.1a]. Collectively, these results provide evidence that Doxorubicin is inducing a prolonged DNA Damage response by continual induction of PARP and p53[Figure 2.1a].
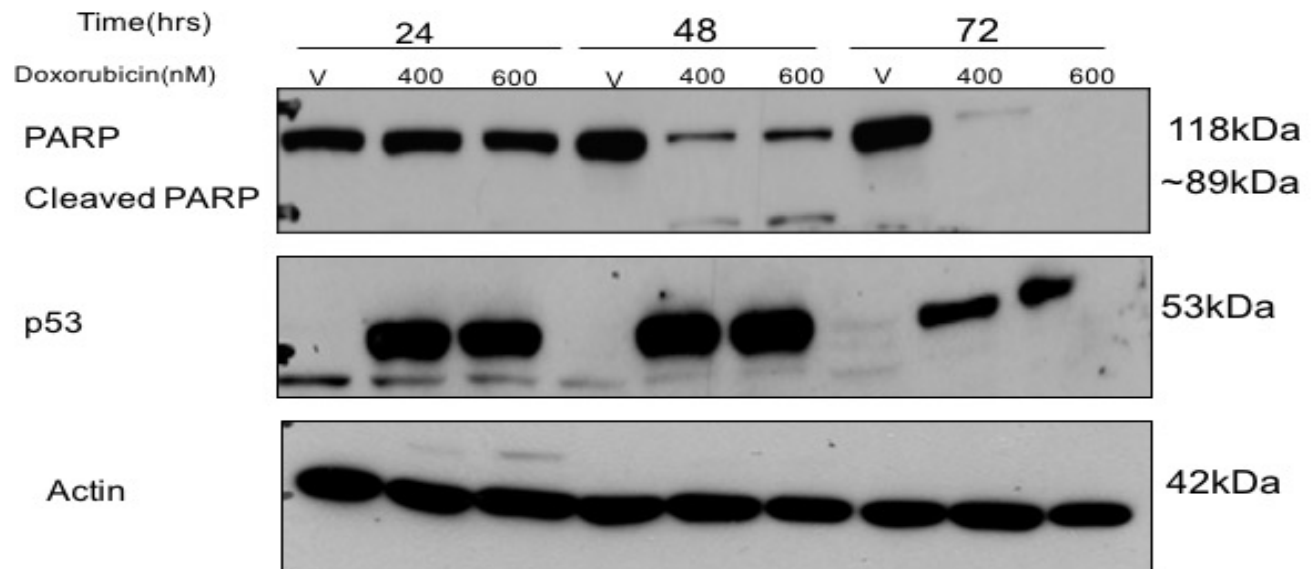
a.)

Figure 2.1. **Sub-Lethal Doxorubicin 24, 48, and 72-hour Time course effect on PARP and p53.**

a.) MCF-7 cells were plated in 60 mm dishes and treated for 24,48, and 72 hours with either DMSO(Vehicle)or sub-lethal doxorubicin, respectively 24, 48, and 72 hours later after treatment, cells were collected and immunoblotted for PARP, p53 and Actin.
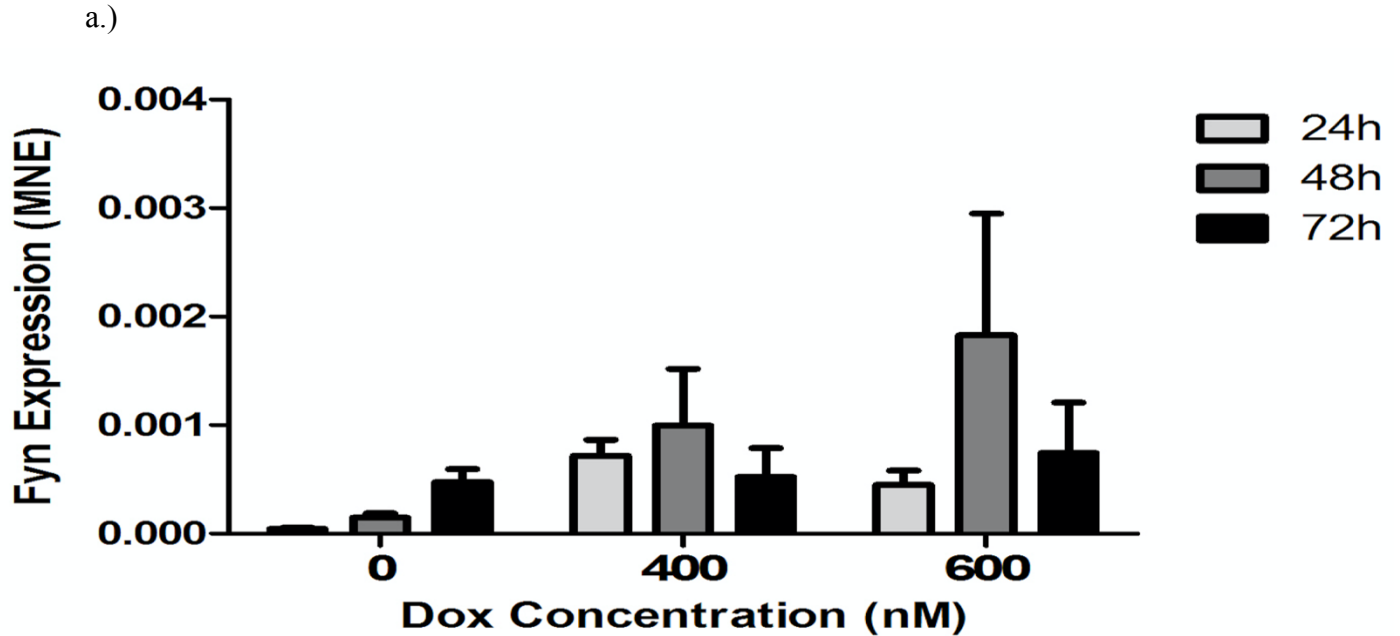
a.)

Figure 2.2. **Sub-lethal Doxorubicin 24, 48, and 72-hour Time course effect on Fyn mRNA expression levels.**

    a.) MCF-7 cells were lysed respectively 24, 48, and 72 hours after treatment with Doxorubicin then RNA was isolated and transformed to cDNA to be quantified by real-time PCR (qRT-PCR) using primers for Fyn and Actin.

Next we looked at the Fyn mRNA induction 24, 48, and 72 hours after sub-lethal Dox treatment [Figure 2.2a]. Our results indicate that Fyn mRNA up-regulation peaked after 48 hours for both 400 and 600nM Dox treated cells, with an 8 and 9.5-fold peak induction respectively. After 72 hours, sub-lethal Dox treated cells had no quantifiable Fyn up-regulation, indicative that Fyn up-regulation is only sustained for 48 hours [Figure 2.2a]. Collectively, these results indicate longer-term sub-lethal Dox begins to promote cell death and down-regulate Fyn mRNA expression. Interestingly, this provides further evidence that a pro-invasive phenotype persists 48 hours after treatment with Doxorubicin.
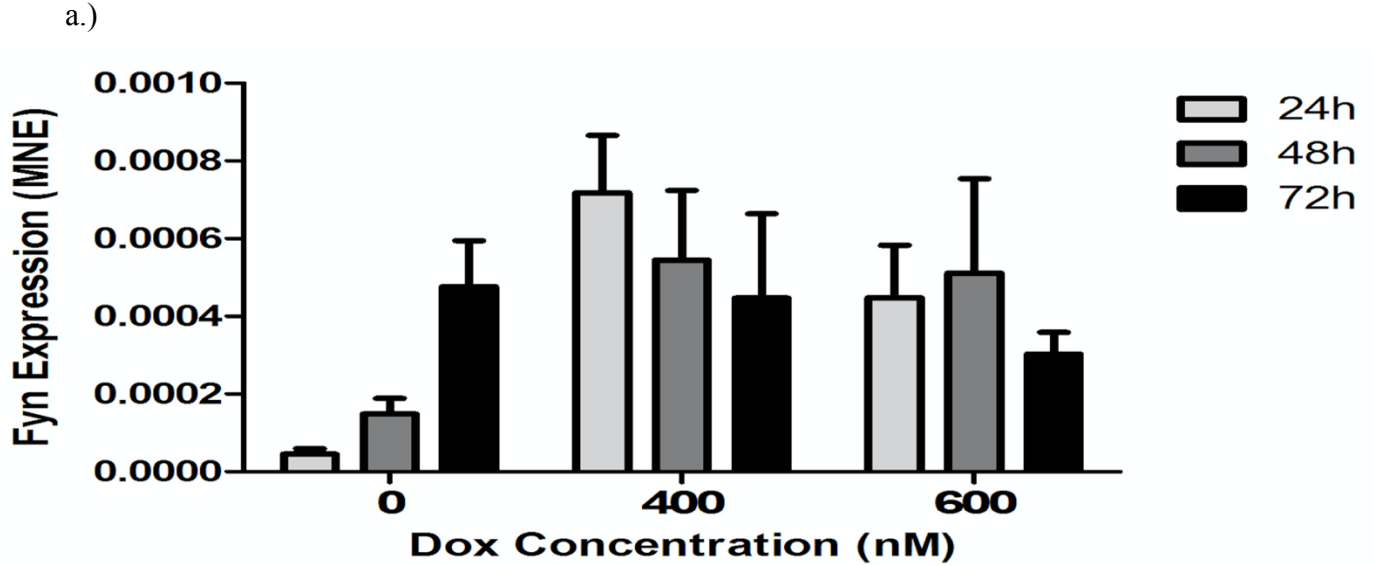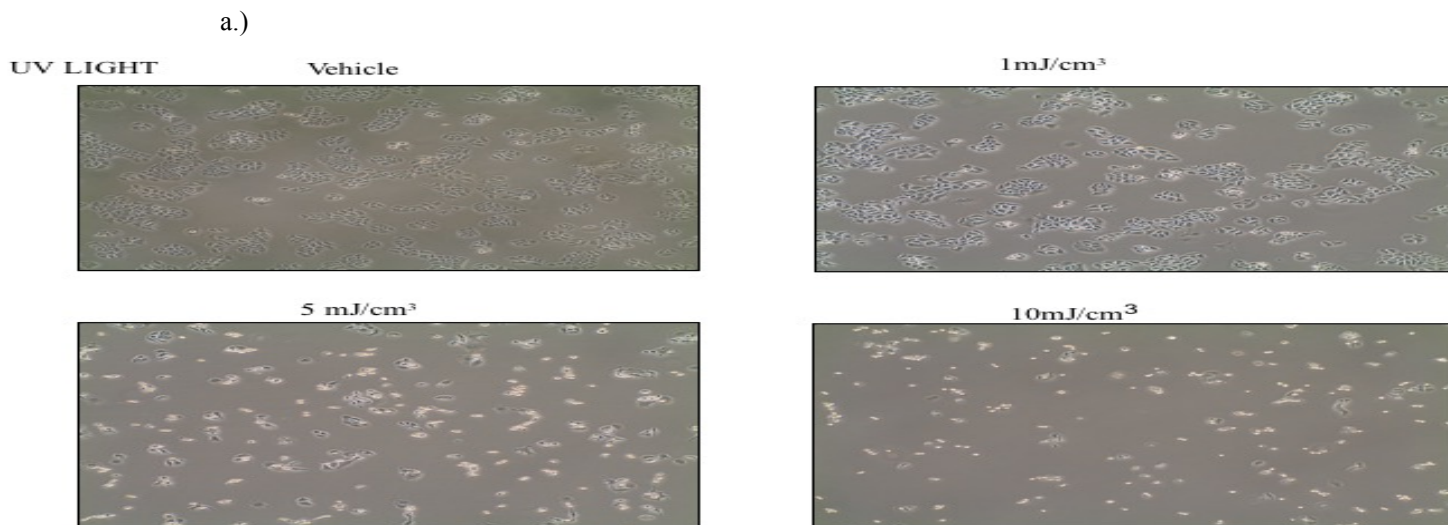
Figure 2.3. Sub-lethal Doxorubicin 24, 48 and 72 *washout effect* on Fyn expression. a.) MCF-7 cells were plated in 60 mm dishes, a set of dishes were treated with V,400, and 600nM Dox for 24 hours. Another set of dishes were washed out with fresh media 24 hours after treatment with Dox and incubated further for 48 and 72 hours. Cells were then harvested and lysed at the indicated time points, then RNA was isolated and transformed to cDNA to be quantified by real-time PCR (qRT-PCR) using primers for Fyn and Actin.
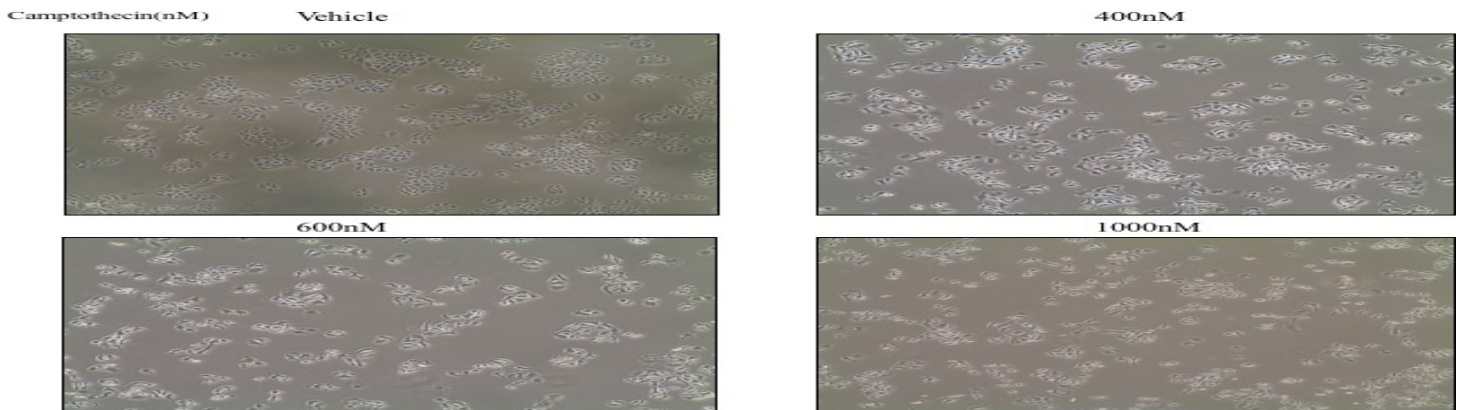
Once we defined the effects of pro-longed sub-lethal Dox on Fyn mRNA, we sought to determine if this effect persisted following Doxorubicin removal. We performed Doxorubicin washout experiments, in which we replaced our initial culture media with fresh media 24 hours after treatment with sub-Lethal Dox. Cells were then harvested at 48 and 72-hour time points, to determine if Fyn is up-regulated in the absence of Dox. Our results indicate that sub-Lethal Dox (400 and 600nM) treated cells that were washout with fresh media had Fyn mRNA induced approximately 50% less compared to cells that did not have Doxorubicin washout [Figure 2.3a]. Collectively, these results suggest Fyn mRNA is still induced following Dox removal, but not to the same extent as with the presence of Doxorubicin, but most importantly these results can suggest a method for clinicians to suppress pro-invasion of Human Breast Adenocarcinoma cells by limiting the amount of time patients are exposed to Doxorubicin [3].

Section 3: Other DNA damaging agents and chemotherapies do not induce Fyn and SRC to the same extent as Doxorubicin.
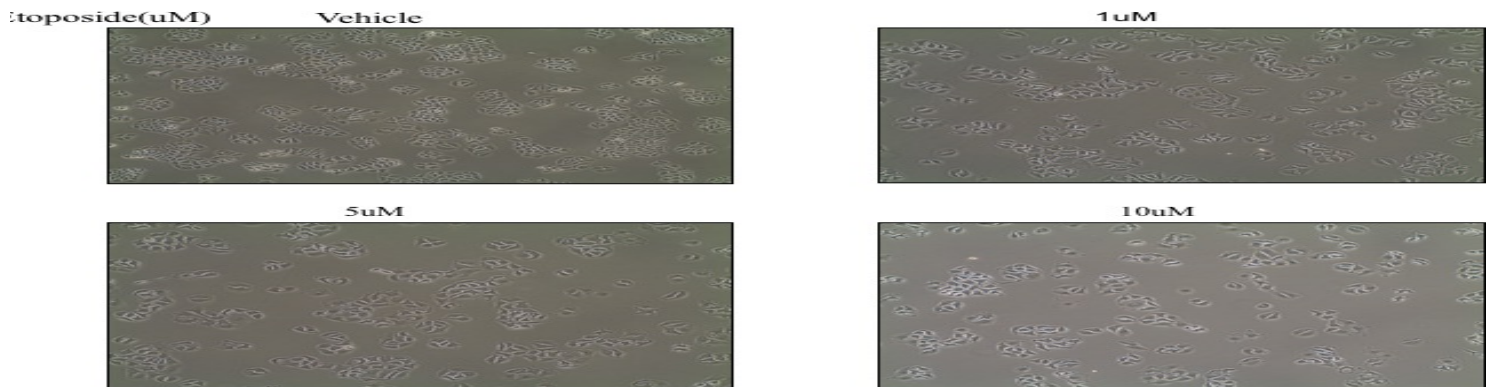
 In order to determine if the up-regulation of SRC tyrosine kinases is a universal mechanism associated with DNA damage agents and/or chemotherapies, we performed a dose response's similar to our Dox dose response. We used commonly used DNA damaging agents and chemotherapeutics such as Etoposide, UV light, Taxol and Camptothecin, to determine if SRC and Fyn are up-regulated to the same extent as Doxorubicin. In addition, we observed the cellular morphology of MCF-7 cells twenty-four hours after treatment with Etoposide UV, Taxol, and Camptothecin [Figure 3.1a, b, c, d]. Our results indicate that MCF-7 cells treated with 1 to 5 mJ/cm^3 of UV light, rapidly decrease in cell number. In addition, cells treated with 10 mJ/cm^3 for 24 hours are no longer detected due to a large increase in cell death [Figure 3.1a]. MCF-7 cells treated with Camptothecin did not show a dramatic decrease in cell number, and likewise cells began to die at 400 and 600nM doses, and cells treated with 1000nM Camptothecin largely decreased in cell number [Figure 3.1d]. In contrast, MCF-7 cells treated with Etoposide and Taxol proved to be more resistant to treatment and showed no significant changes in cell number and morphology [Figure 3.1c, d]. Taken together, these results suggest MCF-7 breast cancer cells have different biological responses to various DNA Damage agents.

a.)

UV LIGHT   Vehicle   1mJ/cm³   5 mJ/cm³   10mJ/cm³

b.)

Camptothecin(nM) Vehicle

400nM

600nM

1000nM

c.)

Etoposide(uM) Vehicle

1uM

5uM

10uM

d.)

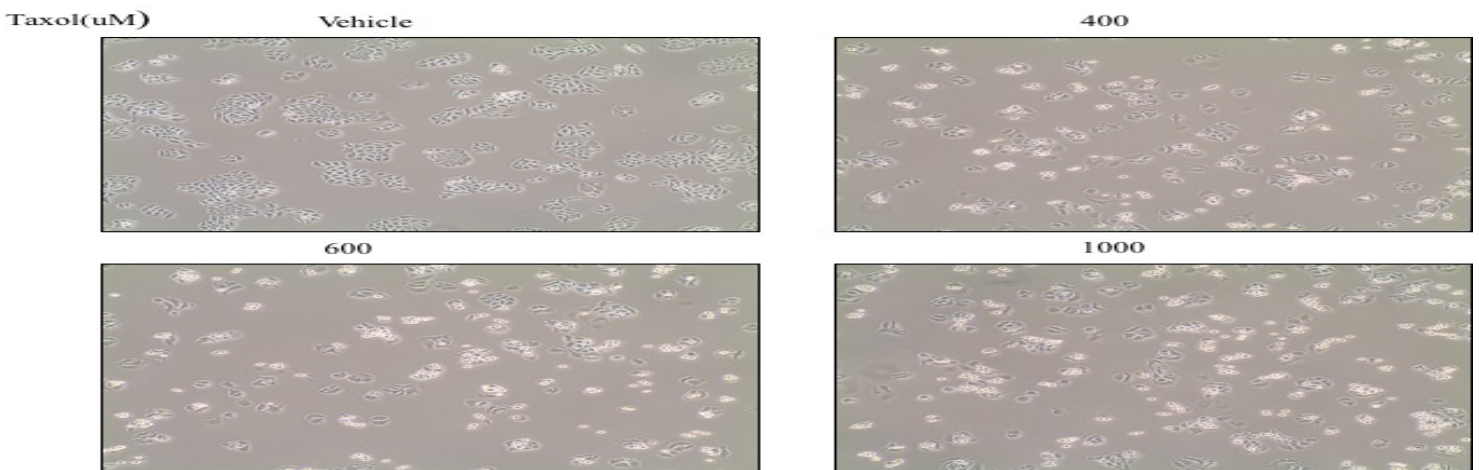Taxol(uM) Vehicle

400

600

1000

Figure 3.1. UV Light, Camptothecin, Taxol, and Etoposide Dose effect on MCF-7 cellular morphology.

a.) MCF-7 cells were seeded in a 60mm dish, cells were treated with Vehicle(DMSO) or UV light for 24 hours, and then cells were viewed under the EVOS microscope prior to collection.

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b.) MCF7 cells were seeded in a 60mm dish, cells were treated with 4:1 Chloroform: Methanol or Camptothecin for 24 hours, and then cells were viewed under the microscope prior to collection.

c.) MCF7 cells were seeded in a 60mm dish, cells were treated with Vehicle(DMSO) or Etoposide for 24 hours, and then cells were viewed under the microscope prior to collection.

d.) MCF7 cells were seeded in a 60mm dish, cells were treated with Vehicle(DMSO) or Taxol for 24 hours, and then cells were viewed under the microscope prior to collection.
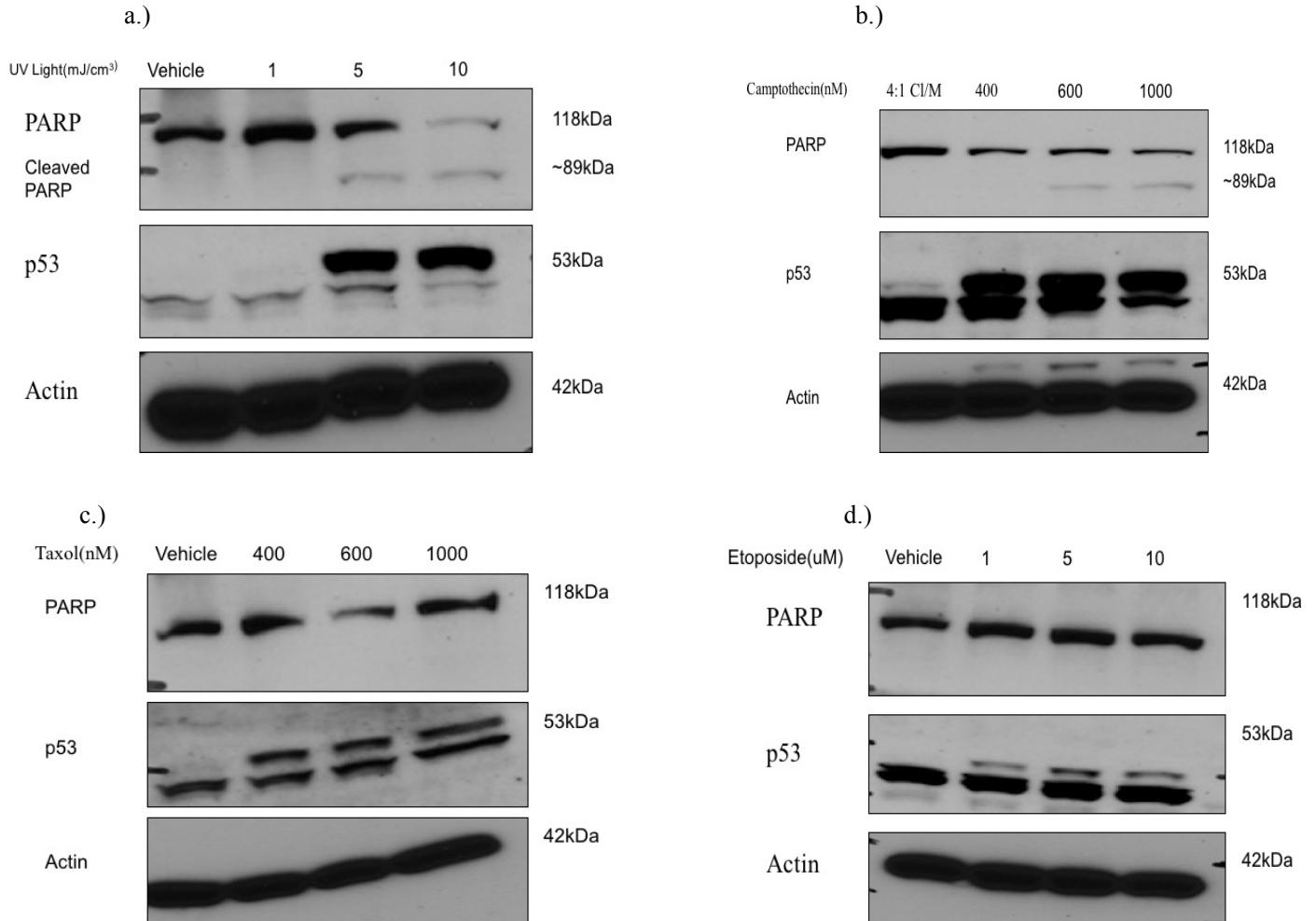
a.)

b.)

c.)

d.)

Figure 3.2. UV Light, Etoposide, Taxol, Camptothecin dose effect on PARP and p53.

a.) MCF-7 cells were seeded in 60 mm dishes and exposed to UV light, and cells were collected after 24 hrs and immunoblotted for PARP, p53 and Actin

b.) MCF-7 cells were seeded in 60 mm dishes and treated for 24 hours with either 4:1 Chloroform/methanol or Camptothecin. After treatment cells were collected and immunoblotted for PARP, p53 and Actin

c.) MCF-7 cells were seeded in 60 mm dishes and treated for 24 hours with either DMSO or Taxol. After treatment cells were collected and immunoblotted for PARP, p53 and Actin.

d.) MCF-7 cells were seeded in 60mm dishes and treated for 24 hours with either DMSO or Etoposide. at different doses. After treatment cells were collected and immunoblotted for PARP, p53, and Actin.

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We initially evaluated PARP, and p53 via western blot to determine the sub-lethal and lethal doses, for these DNA damage agents. For our Etoposide dose response [Figure 3.2d.], p53 was induced with 5uM Etoposide treatment, confirming a genome-wide DNA damage response, but PARP cleavage was not observed at the various Etoposide concentrations, indicating these doses are sub-lethal. For MCF-7 cells exposed to UV light, p53 was induced at 5mJ/cm[3,] in addition to PARP cleavage, indicative of a genome-wide DNA damage response and apoptosis [Figure 3.2a]. For MCF-7 cells treated with Campothecin, p53 was induced at 400nM and PARP cleavage was seen at 600nM, indicative that the sub lethal dose for Camptothecin is 400nM and that the apoptotic dose is 600nM [Figure 3.2]. Lastly, MCF-7 cells treated with Taxol [Figure 3.2c], induced p53 at 400nM concentration, and PARP cleavage was not observed within the 400 to 1000nM dose range. Next we looked at SRC protein induction via western blot for the various DNA damage/chemotherapeutic agents, to determine if induction of SRC protein is DNA Damage or Doxorubicin specific. Our results indicate that SRC protein levels in MCF-7 cells are consistent throughout treatment with Etoposide, UV light, Camptothecin and Taxol, and that induction of SRC protein is Doxorubicin specific [Figure 3.3a, b, c, d].
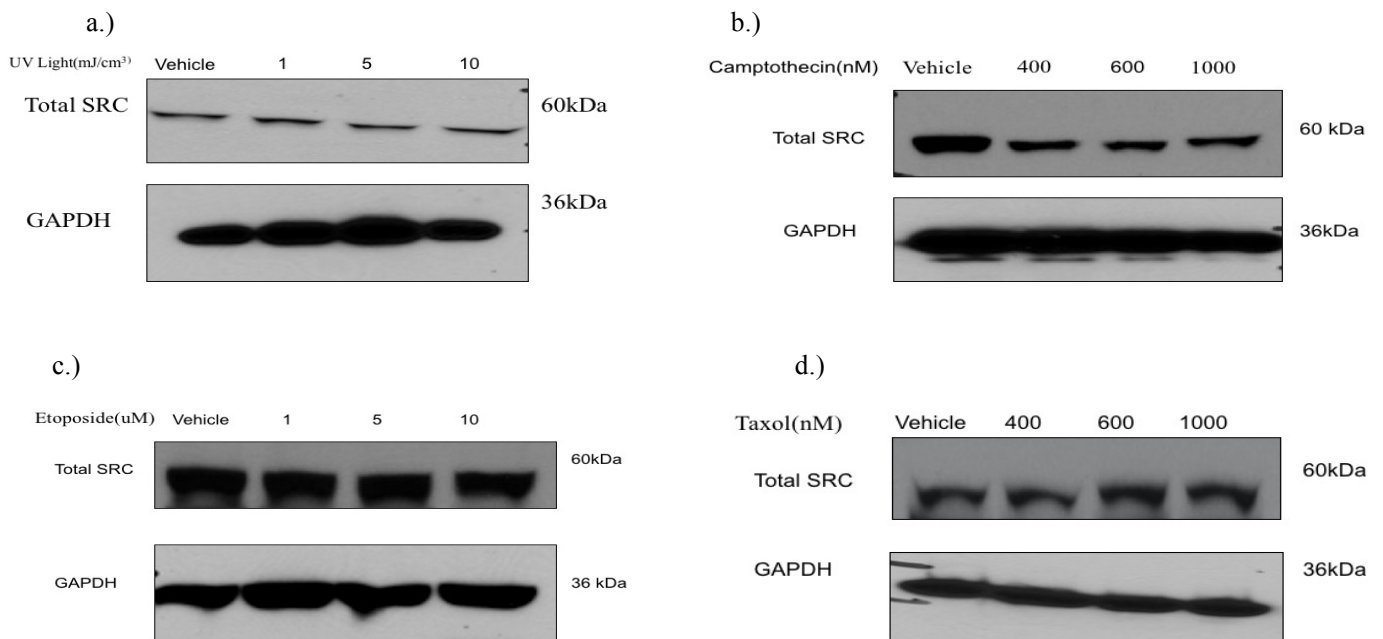

Figure 3.3. DNA Damage/Chemotherapeutic agents effect on SRC protein.

a.) MCF-7 cells were seeded in 60 mm dishes, cells were exposed to UV light for 10 seconds and grown for 24 hours, and then cells were collected and immunoblotted for Total SRC and GAPDH.
b.) MCF-7 cells were seeded in 60 mm dishes and treated for 24hours with either 4:1 Chloroform/methanol or Camptothecin at different doses. After treatment cells were collected and immunoblotted for Total SRC and GAPDH.

c.) MCF-7 cells were seeded in 60 mm dishes and treated for 24hours with either DMSO or Etoposide at different doses. After treatment cells were collected and immunoblotted for Total SRC and GAPDH.

d.) MCF-7 cells were seeded in 60 mm dishes and treated for 24hours with either DMSO or Taxol at different doses. After treatment cells were collected and immunoblotted for Total SRC and GAPDH.

In order to further examine if induction of Fyn mRNA is a universal mechanism associated with DNA damage agents, we first explored the effect of 24 hour UV-light treatment in MCF-7 cells and on induction of Fyn mRNA. Our results indicate that there is a peak 1.75-fold induction of Fyn mRNA after 1mJ/cm^3 of UV light treatment [Figure 3.4a], and no significant quantifiable Fyn mRNA induction after 5 and 10 mJ/cm^3 UV light treatment. We also looked at Etoposide's effect on MCF-7 cells and Fyn mRNA induction 24 hours after treatment [Figure 3.4b].Our results indicate Fyn mRNA is upregulated approximately 2.7, 3.8 and 4.6-fold after treatment with Etoposide Doses of 1 , 5, and 10 μM. In addition, we looked at Camptothecin's effect on Fyn mRNA 24 hours after treatment. Our results indicate that Fyn mRNA is induced 4.1, 4.4, and 2.65-fold with Camptothecin doses of 400 ,600, and 1000 nM [Figure 3.4c]. Lastly, we looked at Taxol's effect on Fyn mRNA 24 hours after treatment, and our results indicate that there is a peak 2.5 -fold induction of Fyn mRNA following 600nM treatment of Taxol [Figure 3.4d]. Collectively, these results suggest that these DNA damage agents also induces Fyn mRNA but not to the same extent as Doxorubicin. Most importantly, they suggest that DNA Damage to human breast adenocarcinoma upregulates pro-invasive genes such as Fyn. Taken together, these results propose that further *in Vivo* studies need to be performed with Doxorubicin to see if the same 17 and 21 fold Fyn mRNA up-regulation is seen in a more physiologically relevant environment.
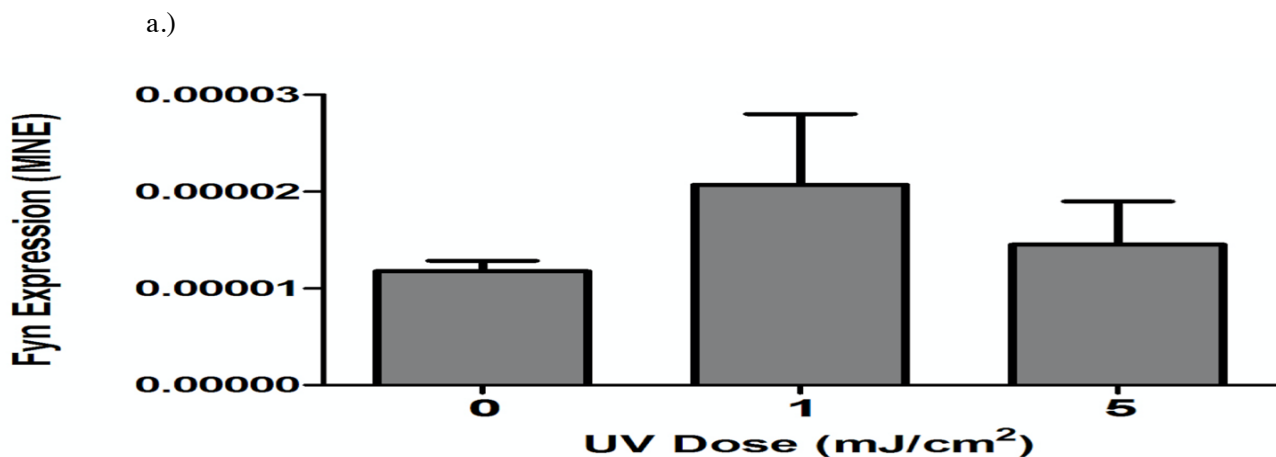
a.)

Figure 3.4a. **UV Light Dose effect on Fyn mRNA.**
a.) MCF-7 cells were lysed 24 h after treatment with UV light. RNA was isolated and transformed to cDNA to be quantified by quantitative real-time PCR (qRT-PCR) using primers for Fyn and Actin.
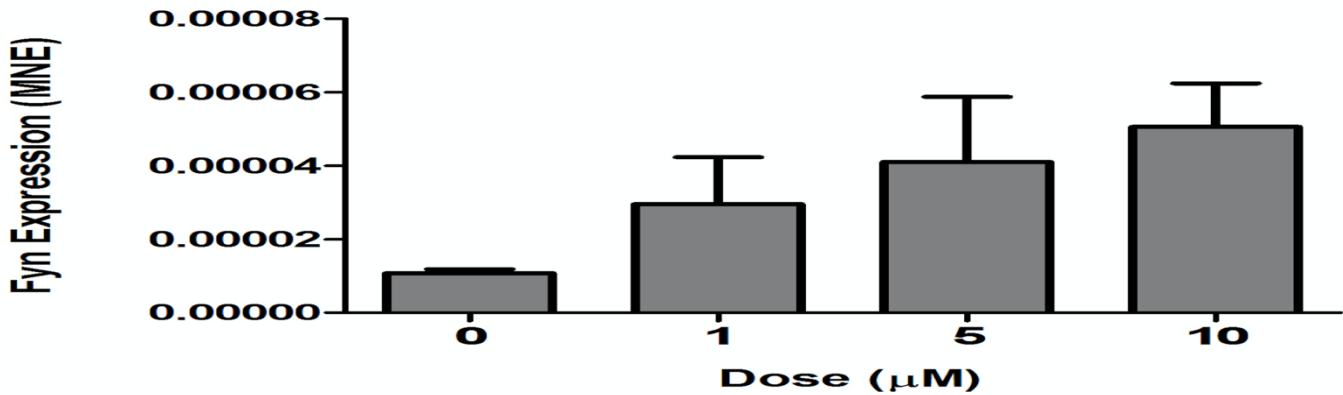
b.)

Figure 3.4b. **Etoposide Dose effect on Fyn mRNA.**

b.) MCF-7 cells were lysed 24 h after treatment with Etoposide. RNA was isolated and transformed to cDNA to be quantified by quantitative real-time PCR (qRT-PCR) using primers for Fyn and Actin.
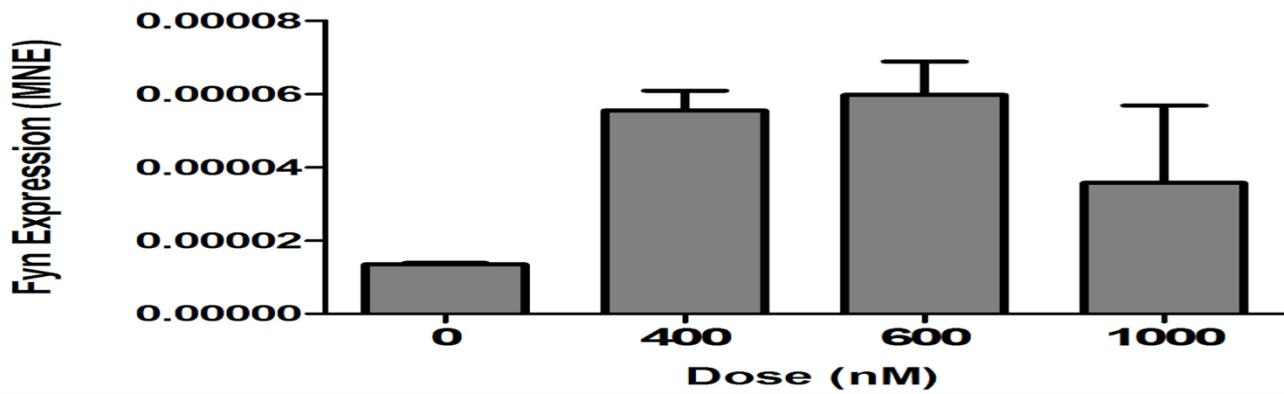
c.)

Figure 3.4c. **Camptothecin Dose effect on Fyn mRNA.**

c.) MCF-7 cells were lysed 24 h after treatment with Camptothecin. RNA was isolated and transformed to cDNA to be quantified by quantitative real-time PCR (qRT-PCR) using primers for Fyn and Actin.

d.)
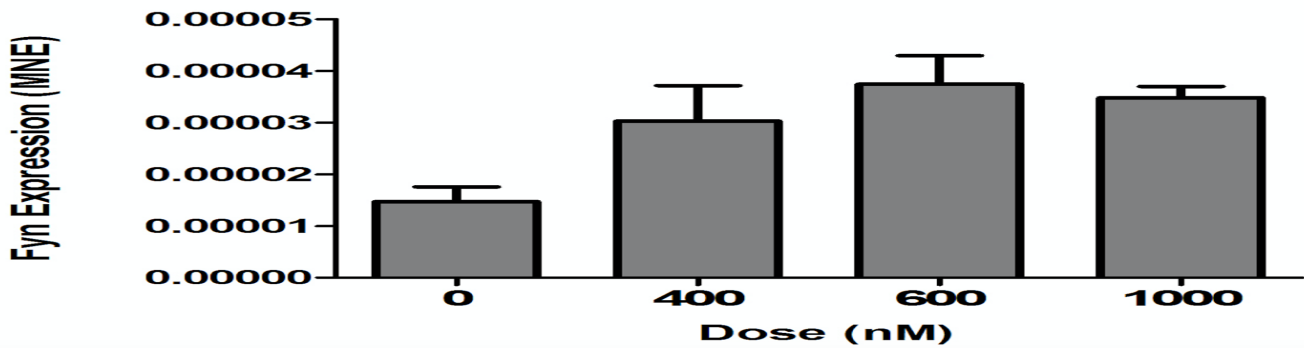

Figure 3.4d. **Taxol Dose effect on Fyn mRNA.**

d.) MCF-7 cells were lysed 24 h after treatment with Taxol. RNA was isolated and transformed to cDNA to be quantified by quantitative real-time PCR (qRT-PCR) using primers for Fyn and Actin.

Section 4: Doxorubicin induces Fyn through p53 but not through induction of EMT (Epithelial to Mesenchymal transition)

Under normal circumstances, p53 is kept at low concentrations in a cell, but in response to stressful cellular events such as DNA double-strand breaks, p53 is activated. Consequently, this leads to a rapid increase in the level of p53 protein and a system of enzymes to repair the damage. We sought to determine if up-regulation of Fyn and SRC is downstream of p53 activation. Our results indicate that cells treated with p53 siRNA and 600nM Dox have a significantly lower level of Fyn mRNA expression in comparison to cells treated only with 600nM Dox [Figure 4.1c]. We also looked at the effect of Chk1 siRNA on sub-lethal Doxorubicin treated cells [Figure 4.1b.], since previous studies have shown that Chk1 is an effector kinase that is activated in response to DNA Damage. Our results indicate that 600nM Dox treated cells induce Fyn mRNA 6 fold with Chk1 siRNA treatment suggesting that transcriptional activation of Fyn mRNA is not inhibited by Chk1 siRNA [Figure 4.1c]. Effective knockdown of p53 and Chk1 were validated via Western blot, [Figure 4.1a, b]. In addition, previous studies have shown that Chk1 protein decreases following Doxorubicin treatment since Chk1 is normally constitutively high in MCF-7 breast cancer cells [33] [Figure 4.1b]. Collectively, these results place Fyn as a downstream transcriptional target of p53 and not Chk1[Figure 4.1c].

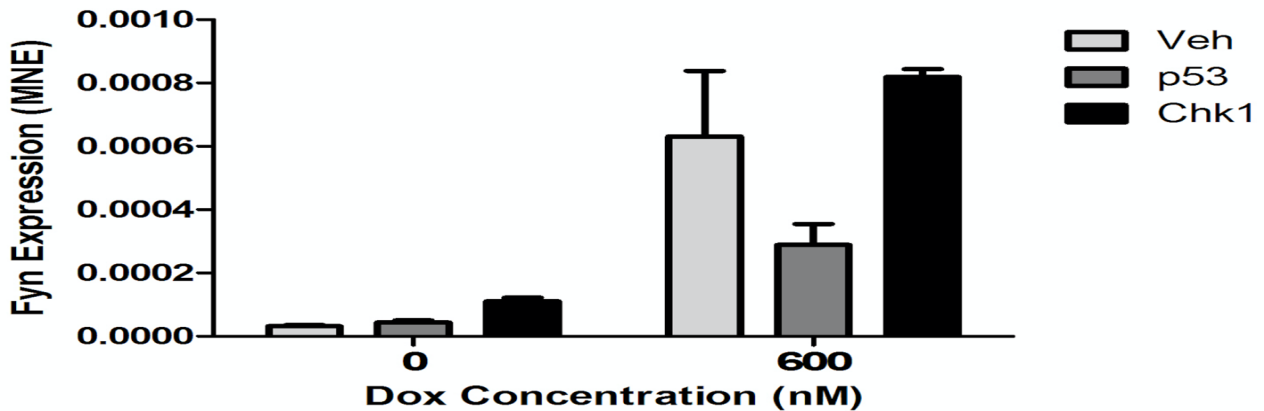a.)   b.)
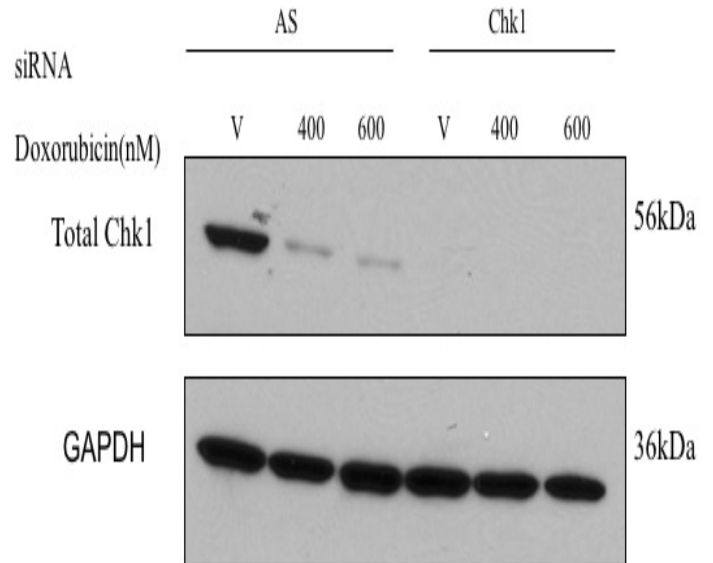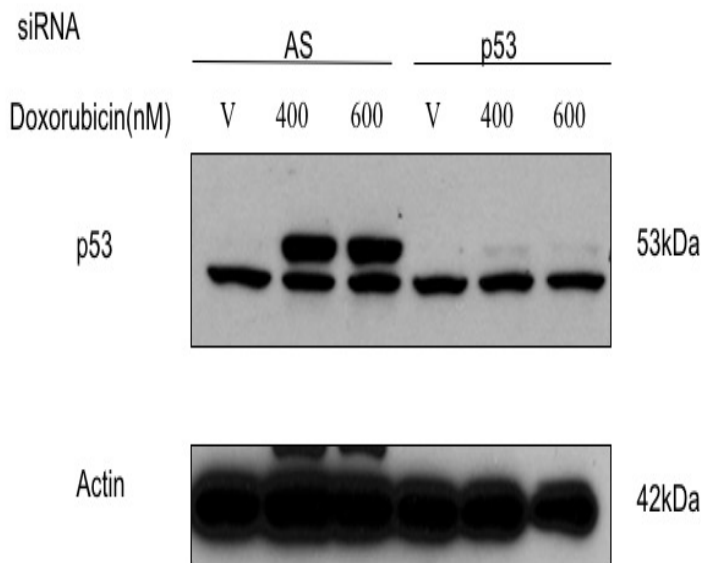
c.)

Figure 4.1. 600nM Sub-Lethal Doxorubicin induction of Fyn is p53 dependent and Chk1 independent.

a.) MCF-7 cells were seeded in 60mm dishes and transfected with siRNA to All Stars Negative Control(AS), and p53. After 24 hours, cells were treated with vehicle, 400nM, and 600nM doxorubicin. After 24 hours' cells were collected and immunoblotted for p53 and Actin.

b.) MCF-7 cells were seeded in 60mm dishes and transfected with siRNA to All Stars Negative Control(AS), and Chk1. After 24 hours, cells were treated with vehicle, 400nM, and 600nM doxorubicin. After 24 hours' cells were collected and immunoblotted for Chk1 and GAPDH.

c.) MCF-7 cells were lysed 24 h after treatment. RNA was isolated and transformed to cDNA to be quantified by quantitative real-time PCR (qRT-PCR) using primers for Fyn and Actin.
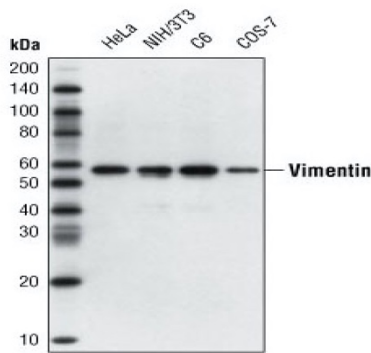
a.)   b.)
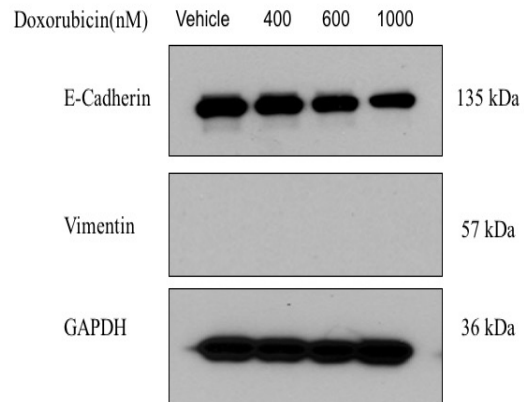
Figure 4.2. Doxorubicin Dose effect on EMT (Epithelial to Mesenchymal transition) Markers.

a.) Western Blot of various cancer cell lines in which Vimentin is induced following EMT. Image obtained from Cell signaling Technologies [3]

b.) MCF7 cells were seeded in 60 mm dishes, dishes were stimulated with Vehicle or doxorubicin, Twenty-four hours after treatment, cells were collected and immunoblotted for E- Cadherin, Vimentin and GAPDH.

Given that sub lethal Dox is inducing a pro-invasive phenotype, it was important to determine if this is associated with an EMT in MCF-7 breast cancer cells. When breast cancer cells become more aggressive, they often undergo an EMT. Cells that have

undergone an EMT have lost epithelial markers such as *E-cadherin* and gained mesenchymal markers such as Vimentin [Figure 4.2a], and tend to be more invasive and resistant to chemotherapies [34]. Numerous studies have described a partial or complete loss of E-cadherin during carcinoma progression, which is correlated with an unfavorable prognosis. Based on our results, sub-lethal Dox did not induce an EMT, since epithelial marker E-Cadherin only slightly decreased and the mesenchymal marker Vimentin was not induced, indicative that up-regulation of pro-invasive genes is not associated with an EMT. Further investigation is required to determine the specific signaling pathway that is inducing invasiveness in MCF-7 breast cancer cells.

Section 5: **Doxorubicin induces Biologic responses in a SRC/Fyn dependent manner**

Transcriptional upregulation of SRC and Fyn mRNA following sub-lethal Doxorubicin treatment proved to be insightful of the biological behavior of MCF-7 breast cancer cells. Our 2-D images show that MCF-7 cell's confluence is disrupted at 400 to 600nM Doxorubicin doses [Figure 5.1a]. Collectively, our results suggest that sub-lethal Doxorubicin is disrupting cell to cell contacts, thus allowing cells to become more migratory and invasive. In addition, these results can provide the groundwork for future experiments that look at invasion and metastasis of MCF-7 breast cancer cells in an *in vivo* environment.
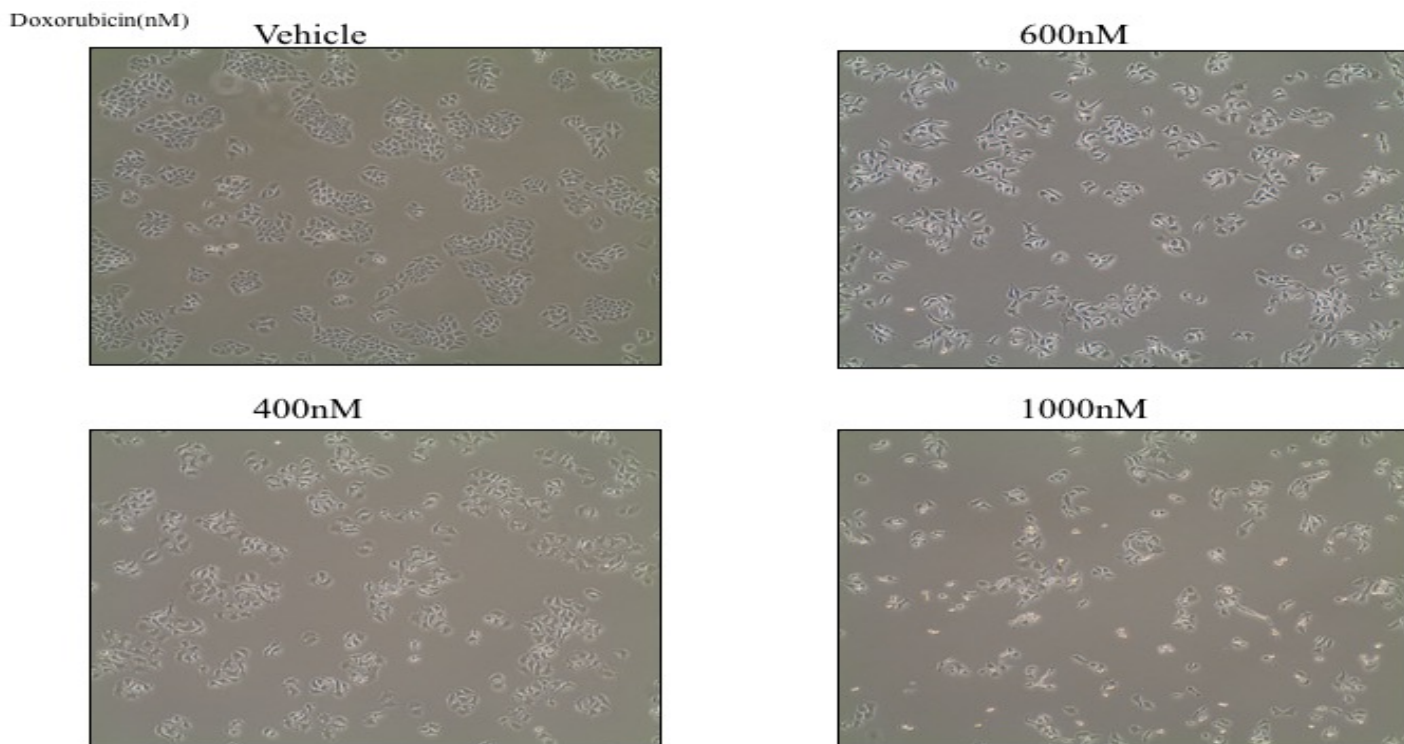
a.)

Doxorubicin(nM)

Vehicle   600nM

400nM   1000nM

Figure 5.1. **Doxorubicin Dose effect on 2-D mono-layer MCF-7 cell morphology.**

a.) MCF-7 cells were seeded in a 60mm dish, cells were treated with Vehicle or Doxorubicin for 24 hours, and then cells were viewed under the EVOS microscope prior to harvesting.
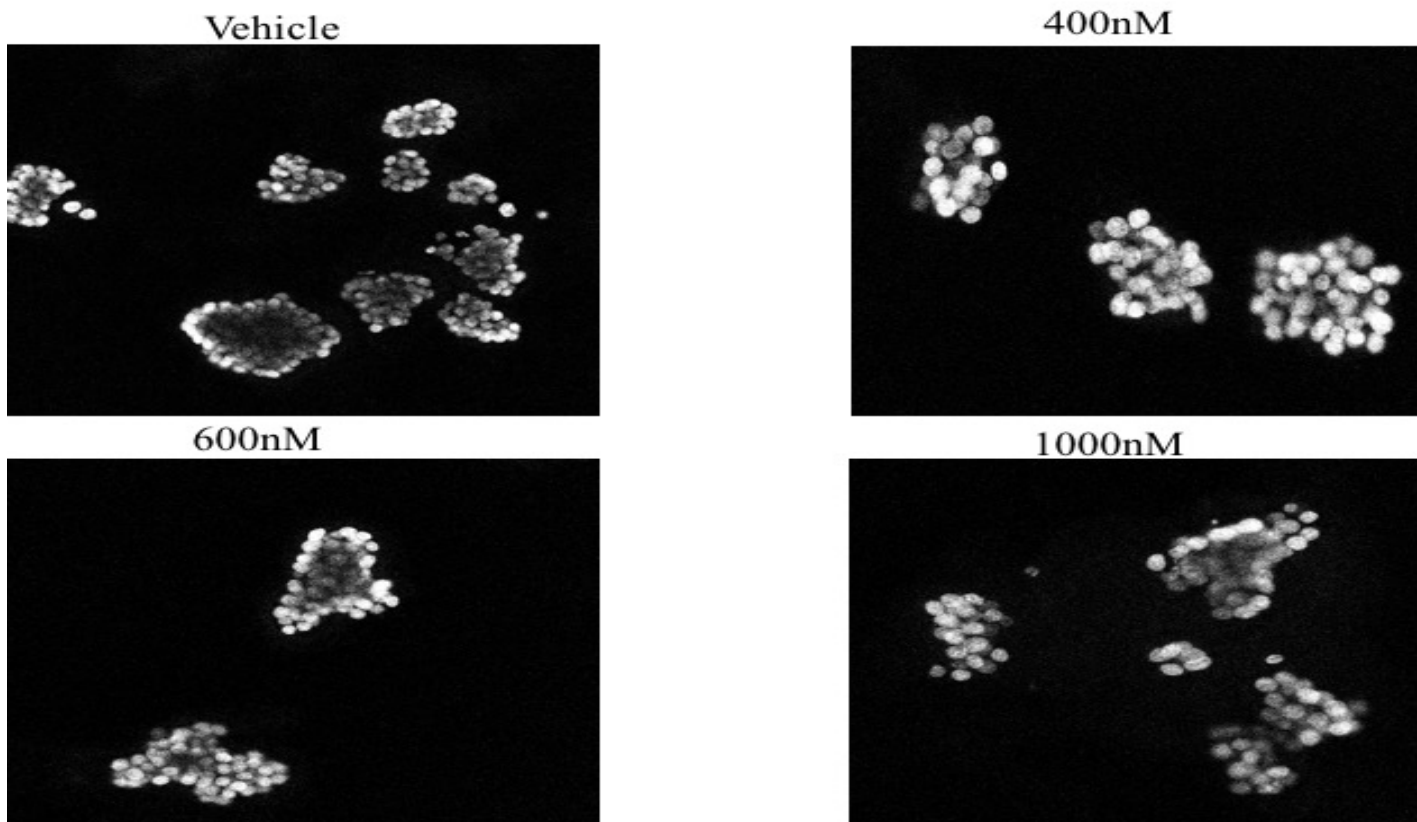a.)

Figure 5.2. **3-D Culture of MCF-7 cells with DAPI Stain.**

a.) DAPI-Stained images of MCF-7 cells grown in 3D culture using matri-gel as the extracellular matrix. Confocal Microscope was used to observe histological changes following Doxorubicin treatment.

In order to have a more physiologically relevant representation of human breast epithelial cancers, we grew MCF-7 cells in 3D culture to observe biological events that may be associated with Doxorubicin treatment. Our first image shows MCF-7 cells that have not been treated with Doxorubicin, and subsequent treatment with 400nM Dox shows a slight disruption in spherical morphology. Cells treated with 600nM Dox begin to display a grape-like morphology and cells treated with 1000nM Dox are all in grape-like clusters. Our 3D Culture results indicate that Doxorubicin is causing architectural disorders and nuclear pleomorphisms in MCF-7 alongside changes in SRC and Fyn gene expression [previous results]. Further studies need to be done in order to determine the sub-lethal and lethal Doxorubicin dose for this 3D microenvironment [12].

a.)
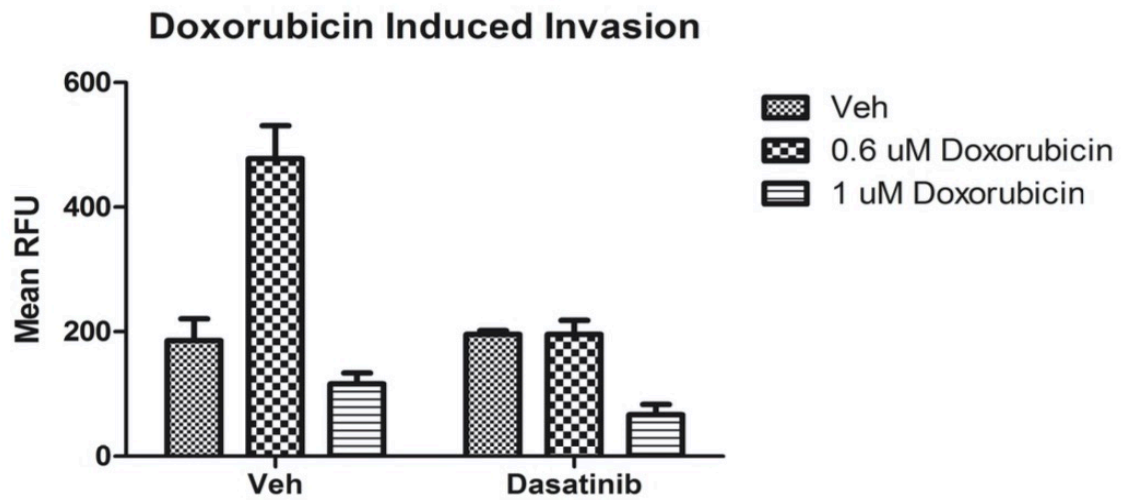
Figure 5.3. **Effect of Dasatinib on Pro-Invasive phenotype** [Achraf Ali Shamseddine and Benjamin Joseph Newcomb Data] [32].

a.) MCF-7 cells were treated with +/- Doxorubicin and/or with +/- Dasatinib.

Dasatinib, a SRC family kinase inhibitor, was used in conjunction with Doxorubicin to determine if pro-invasive genes such as SRC and Fyn were still up-regulated after Dasatinib treatment. Our Newcomb and Shamseddine results show that sub-lethal Dox increases invasion and that Dasatinib reverses that effect [Figure 5.3.]. Collectively, a combination treatment of Doxorubicin and Dasatinib, may be a more effective treatment to block the growth and invasion of breast cancers [13].

Discussion

In this study, we investigated the hypothesis that low doses of doxorubicin induce SRC and other tyrosine kinases through specific activation of DNA damage pathways in MCF-7 breast cancer cells. We initially confirm the doses of 400 and 600nM Doxorubicin are sub-lethal at least at earlier time points, by probing these various doses for PARP cleavage, Since PARP is specifically cleaved during apoptosis, this allowed us to confirm that the Doxorubicin doses above are sub-lethal since no PARP cleavage was observed. Similarly, we probed for p53 via western blot in order to confirm that sub-lethal Doxorubicin is inducing a genome-wide DNA damage response by up-regulating p53 protein levels in MCF-7 breast cancer cells.

Our study indicates that sub-lethal Doxorubicin primarily induces Fyn at the transcriptional level with little to no further induction seen at the translational level [Figure 1.4a]. Previous studies have shown that Fyn and other SRC family kinases tend to be overexpressed in breast, colon and thyroid cancers [6]. Collectively, our data sheds light on genes that may induce cancer cell invasion as a result of DNA Damage through sub-lethal Doxorubicin treatment.

Clinical studies done by the Cancer and Leukemia Group B(CALGB) on patients with stage 2 node positive breast carcinoma demonstrate a difference in relapse and survival for patients treated with different chemotherapy doses [3]. Patients that receive low dose chemotherapeutics had higher rates of primary tumor relapse then patients treated with higher doses. This group concluded that doses of chemotherapy should not be reduced, in order to maximize overall survival. Although, increasing the chemo-therapeutic dose suppresses cancer cell growth, the specific genes and/or signaling pathways that are dysregulated are not specifically targeted and may eventually lead to the recurrence of a breast carcinoma.

Multiple *in vitro* studies have shown that cancer cells exposed to anti-tumor drugs such as Doxorubicin may be directly induced to express a subset of genes that can confer resistance, in addition, transient treatment with Doxorubicin may alter the expression of a diverse group of genes in a time-dependent manner [22]. We explored if these phenomena hold for activation of SRC family kinases by performing time course experiments [Result Section 4]. Interestingly, MCF-7 breast cancer cells treated with sub-lethal Doxorubicin for 48 hours had an acute and transient up-regulation of Fyn mRNA [Figure 2.2a]. In addition, cells that had sub-lethal Doxorubicin removed after 24 hours did not have the same sustained Fyn mRNA upregulation [Figure 2.3a]. Collectively,

sustained up-regulation of Fyn mRNA may be representative of a pro-invasive signature profile of doxorubicin, and may contribute to resistance of breast cancer cells as described above [22].

In addition to Doxorubicin, we used other chemotherapeutics such as UV light, Camptothecin, Etoposide, and Taxol to examine the pro-invasive signature we define above. Treatment of MCF-7 cells with these other chemotherapy agents did not induce Fyn to the same extent as Doxorubicin [Figure 3.4]. In addition, there were no changes in SRC protein following UV light, Camptothecin, Etoposide and Taxol treatment [Figure 3.3]. Collectively, these results provide evidence that DNA damage is inducing Fyn mRNA and as described previously and this up-regulation of Fyn may be a universal response of cancers cells, to anti-tumor and DNA damage agents. Defining the exact biological changes Fyn is inducing is worth exploring, given that previous studies have shown Fyn to contribute to tumor progression [11].

Given that sub lethal Dox is inducing pro-invasive genes, it was important to determine if this is associated with an EMT in MCF-7 cells. Cells that have undergone an EMT tend to be more invasive and resistant to chemotherapies. Numerous studies have described a partial or complete loss of E-cadherin during carcinoma progression, which is correlated with an unfavorable prognosis. Our results indicate sub-lethal Doxorubicin is not inducing Vimentin a mesenchymal marker, and further confirm that sub-lethal Doxorubicin is up-regulating Fyn through p53 and a DNA damage response.

We viewed MCF-7 breast cancer cells under the EVOS and Confocal Microscope to observe its biological behavior after sub-lethal doxorubicin treatment [Section 5]. Specifically, we used 3D cell cultures to have deeper insights of cell adhesion and migration in MCF-7 cells. Our results indicate sub-lethal doxorubicin is disrupting cell to cell adhesion with increasing Dox doses, [Figure 5.2]. Future experiments using loss of function mutations for Fyn in a 3D culture setting, will further validate any alterations in biological behavior after sub-lethal doxorubicin treatment.

In order to determine the specific DNA damage pathway that is activated in response to sub-lethal Doxorubicin, we used siRNA for p53 and Chk1, in order to determine if they are required for Fyn mRNA induction in response to Doxorubicin. Chk1 and p53 proteins are normally activated in response to DNA damage as a protective mechanism to halt cell growth and repair damage [21]. We found that inhibition of p53 suppressed the up-regulation of Fyn mRNA. However, inhibition of Chk1 had no effect on Fyn mRNA up-regulation. Collectively, our results

place Fyn downstream of p53. Further investigation of the potential roles of Fyn and its role in pro-invasion following sub-lethal doxorubicin treatment in MCF-7 breast cancer cells can potentially provide a viable therapeutic approach for patients with stage 2 estrogen-receptor positive breast cancer.

In our study all of our experiments were done using *in vitro* systems. Future experiments would involve the use of animal models to study pro-invasion in a more physiologically relevant environment. We can initially perform a Xenograft of MCF-7 cells in NOD/SCID mice until a palpable tumor is found then treat the mice with sub-lethal Dox, and remove the tumor to perform immunohistochemistry for Fyn and other pro-invasive proteins. This can initially tell us if up-regulation of Fyn is occurring *in vivo* following Doxorubicin treatment.

In addition, mechanistic studies such as 3D Invasion assays, will allow us to study the migration of MCF-7 breast cancers in 3D after Doxorubicin treatment. To perform this assay, we would use a trans-well chamber with a membrane coated with extracellular matrix. to observe the migration of MCF-7 cells through the trans well after Dox treatment. This can give us a better idea of the invasive behavior, and it allows us to observe cancer metastasis using a 3D micro environment [20].

In conclusion, this study identifies Fyn as the major SRC family tyrosine kinase that is upregulated in response to doxorubicin, a first-line chemotherapy agent used in the treatment of Human Breast Adenocarcinoma, and suggests that Fyn is inducing invasion of MCF-7 cells. Moreover, Fyn mRNA induction is dependent on p53, placing Fyn as a major transcriptional target of the DNA damage pathway. Further investigation of the probable roles of Fyn upregulation as a result of DNA damage can enhanced understanding of the biological relevance of its activation, as well as the potential benefit of inhibiting Fyn in conjunction with chemotherapeutic treatments.

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