AMERICAN UNIVERSITY OF BEIRUT

EFFECTS OF SUB-LETHAL HIGH INTENSITY FOCUSED ULTRASOUND (HIFU) EXPOSURE ON MAMMARY EPITHELIAL TUMORIGENESIS AND CYTOTOXIC RESPONSE TO ANTI-NEOPLASTIC AGENTS

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biology
of the Faculty of Arts and Sciences
at the American University of Beirut

Beirut, Lebanon
November, 2014

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Date of thesis/dissertation defense: November 25, 2014

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ACKNOWLEDGMENTS

I was thinking of how to start this very precious part. I couldn't find better than this Vietnamese Proverb: "When eating a fruit, think of the person who planted the tree". This is a dedication to every person who helped me cross this bridge to my next destination.

First and foremost, I would like to thank you Dr. Diana for your tremendous help during the past couple of years. I have come a long way since my first day in this program thanks to your valuable advice. You have shown me what it means to be a successful dedicated woman. I have felt your warmth and your amazing spirit the first day I came to your office and I have never imagined I would get to know one of the most brilliant scientists.

Dr. Talhouk and Dr. Zeidan, thank you for accepting to serve on my thesis committee. Your remarks and your approach to science from the brief encounters we shared helped me get on the right track in my research project.For that and for your continued support and encouragement, thank you so much.

Dr. Oweis, thank you for all the effort you have put to ensure the best combination possible between biology and mechanical engineering. This collaboration is certainly a big gain to us. Hussein, thank you for all the time you have put during experiments and optimization procedures. Finding the focal point will always be one of the hardest and challenging things that I ever had to do: p

Dr. Doris, thank you for being the launching point for my journey and, for being the amazing teacher that I will never forget.

Dima and Hind, you are truly the best lab members and friends one could ask for. Dima, you have introduced me to the true version of Islam by being the nice, amazing, and caring person you are. Hind, I am really happy I got to know a person like you; so different in character, yet so close in spirit. Carol, thank you for being this incredible person, and friend. I love you girls!!! Sara, Dana, Lara, Lori ¨DJ Lab members¨, I am so fortunate to have met you.

Nisreen, thank you for helping me in my stats I really appreciate it. Thank you for being always here and for answering any questions I had.

Mom and Dad, thank you for the constant support throughout my life. You have helped me be the person I am. Thank you Mollo, "ya habib albe enta", don't forget about your older sister when you become famous ok?:P Bellaa, you know that I wouldn't have done any of this if it weren't for you? You are my angel. I cannot describe what you mean to me. I love

you so much little sister. Zanzouna, thank you for making our stay at grandma’s a blast, and for making my medicine injections a fun thing after all :P

George, Andrew and Samer, you are the BEST male friends a girl could ever have. I love you all so much, thank you for being here when I needed a friend. You have truly added joy to my life.

My soul mate; my all, no words can ever describe what you mean to me ya Wilson. THANK YOU for these 7 wonderful years during which we have grown and matured together.

Finally, I would like to dedicate this work to the soul of MajedZaher. I haven't got the chance to meet you Dear Majed, but I know how much your entourage adores you. Pray for us young angel.

AN ABSTRACT OF THE THESIS OF

Ingrid FaridYounes for Master of Science
Major: Biology

Title: Effects of sub-lethal high intensity focused ultrasound (HIFU) exposure on mammary epithelial tumorigenesis and cytotoxic response to anti-neoplastic agents

High Intensity Focused Ultrasound (HIFU) is a therapeutic modality that is used to destroy unwanted tissues including solid tumors inside the body. At the focal point where the acoustic waves are intensified, cell death can result from cavitation and/or thermal ablation effects. However, the effects of sub-lethal HIFU exposure on cell function remain to be elucidated. Given that HIFU exposure results in pressure/tension waves that can cause cellular deformations, we hypothesize that sub-lethal HIFU treatment could result in mechanotransduction alterations that may alter tumorigenesis of mammary epithelial cells and may modulate their response to anti-neoplastic agents. The objective of this study is to examine the alterations in mechanotransduction due to changes in the physical properties resulting from the exposure of MDA-MB-231 breast cancer cells and MCF-10A immortalized mammary epithelial cells to ultrasonic waves from a custom-designed HIFU setup and to determine consequences on cellular response to anti-neoplastic agents. Combined with data from a previous study, we had assessed the *in vitro* effects of sub-lethal HIFU exposure on the expression of sevenmechanosensitive genesnamely *CAV-1* (Caveolin-1 α & β), *Hic-5* (Hydrogen Peroxide-Inducible Clone 5), *PXN* (Paxillin), *TTLL4* (Tubulin-Tyrosine Ligase-Like Protein 4), TWIST1 (Twist-Related Protein 1), CTSD (Cathepsin D), and *HSPA1A* (Heat Shock Protein 70) whereby we quantified significant enhanced expression of *CAV-1*α, *PXN*, and *Hic-5* that was immediate-early in MCF-10A cells and delayed in MDA-MB-231 Cells. Additionally, we noted an immediate -early transient increase in *TTLL4* expression in both cell lines and in *TWIST1* expression in MDA-MB-231 cells. Notably, sub-lethal HIFU exposure had no significant effect on the expression of *CAV-1*(total pool), *CTSD*, and *HSPA1A* in both cell lines. Moreover, sub-lethal HIFU exposure of cells at 6hr or 30hr prior to the *in vitro* addition ofanti-neoplastic agents sensitized MDA-MB-231 and MCF-10A cells to suboptimal cytotoxic doses of Taxol (1.5nM, 7.5nM) and Doxil (0.05μM, 0.5μM) when tested over four consecutive days. Furthermore, MDA-MB-231 cells surviving single or dual rounds of HIFU exposure at the

focal point and passaged for 3-to-6 weeks in tissue culture show no significant change in their *in vitro* sensitivity to Taxol or Doxorubicin. Future work is intended to determine if sonoporation - among other mechanisms that are related to the above mentioned mechanotransduction alterations - is implicated in the enhanced *in vitro* sensitivity of both cell lines to suboptimal cytotoxic doses of Taxol and Dox post sub-lethal HIFU exposure. Subsequently, we plan to assess post-translational changes in phosphorylation of Cav-1 α, Hic-5, Paxillin, and TTLL4 in both cell lines and to examine if cells exhibit changes in lumen formation and/or cellular polarity in 3-D matrigel post *in vitro* exposure to sub-lethal HIFU.

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ABBREVIATIONS

/	Per
%	Percent
μg	Micro Gram
μl	Micro Liter
˚C	Degrees Celsius
CAV-1	Caveolin-1
CTSD	Cathepsin D
DMEM-F12	Modified Eagle's Medium Ham's F-12
DNA	Deoxyribonucleic Acid
PBS	Phosphate Buffered Saline
ECM	Extracellular Matrix
EGF	Epithelial Growth Factor
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular-Signal-Regulated Kinase
et al.	etallii (and others)
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HeLa	Henrietta Lacks cells

HER	Human Epidermal Growth Factor Receptor
HIFU	High Intensity Focused Ultrasound
hr	Hour
HSP70	Heat Shock Protein 70
Hz	Hertz
INF-α	Interferon Type 1
In Situ	On Site
MCF-10A	Michigan Cancer Foundation cells number 10 A
MCF-7	Michigan Cancer Foundation cells number 7
MDA-MB-231	Monroe Dunaway Anderson Metastatic Breast cancer cells number 231
min	minute
MHC	major histocompatibility complex
mm	Millimeter
MMP	Matrix Metalloproteinase
msec	Millisecond
miR	Micro RNA(mRNA)
P	p-value
P53	Tumor Protein 53
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
RNA	Ribonucleic Acid
rpm	Rounds Per Minute
RPMI	Rose Park Memorial Institute
Sec	Second

Src	Proto-oncogene tyrosine-protein kinase
TNF-α	Tumor Necrosis Factor
TWIST 1	Twist family bHLH transcription factor 1
Vpp	Peak to Peak Voltage
VEGF	Vascular Endothelial Growth Factor

CHAPTER I

LITERATURE REVIEW

A. Mammary Gland

1. Development Processes and Pathways

The mammary gland is composed of different types of cells including adipocytes, vascular endothelial cells, immune cells and stromal cells. The mammary gland is a secretory organ that starts developing during embryonic life, pauses at birth and carries on at puberty (Reviewed by Watson *et al*. 2008).

What we know about the development of this gland is derived from the murine model where its development starts at embryonic day 14 with mammary buds followed by ductal branching and ductal tree morphogenesis at week 16. Simultaneously, the ductal lumen and nipples are formed viamany morphological alterations. Finally, at pregnancy, the milk producing gland will achieve its maturation (Reviewed by Cowin*et al*. 2010). The branching morphogenesis is achieved by the help of many hormones, enzymes and ECM proteins. The most important hormone in this process is estrogen, which activates the proliferation of the primitive duct leading to the terminal end bud (Reviewed by Gjorevski*et al*. 2011).

A proper mammary gland development is regulated by multiple signaling pathwaysespecially during puberty. The major types of pathways implicated in this process include some paracrine growth factors activated by the ovary and the pituitary to regulate

proliferation and branching, someautocrine factors to negatively control mammary gland morphogenesis, and MMPs for the migration and patterning of the gland (Reviewed by Gjorevski*et al*. 2011).

B. Breast Cancer

1. Overview

Breast cancer is one of the most widespread cancers worldwide with more than a million cases and half a million deaths each year (the Cancer Genome Atlas Network, 2012). Its causes are diverse; for instance genetic predisposition, hormonal supplements and viral infections could be the cause of breast cancer emergence. While many factors could lead to breast cancer, only few types surface. These are mainly divided into two parts; the In Situ versus the Invasive. The In Situbreast cancer, the latter remains in the duct (ductal carcinoma) or in the lobules of the breast (lobular carcinoma) while the cancer is considered invasive or infiltrating if the cancer cells penetrate the membranes surrounding the duct (infiltrating ductal carcinoma) or the lobule (infiltrating lobular carcinoma) (Patidar*et al*. 2012).

2. Pathways Implicated In Breast Cancer

a. Proteins and Mutations Involved

Several mutations have been implicated in breast cancer initiation and progression; these could implicate mutations in tumor suppressor genes or proto-oncogenes (reviewed by Castaño*et al*. 2011). Epigenetic changes also play a role in the development of breast

cancer. In fact miRNA analysis in a pool of 76 breast cancer samples identified up to 29 miRNAsimplicated in breast cancer when deregulated such as, miR-145 and miR-155 (reviewed by Singh and Mo.2013). Moreover, breast cancer could be developed by many molecules that function at the level of cell proliferation or survival. This is the case of the HER family that is involved in many processes leading to cell survival among other biological processes. Additionally, it has been shown that many members of HER family such as HER-1 and HER-2 are expressed in breast cancer and are associated with poor prognosis in patients. On the other hand, any deregulation in apoptosis could also lead to tumor development by helping cancer cells escape death hence, becoming more resistant to therapeutic modalities. Another factor implicated in breast cancer development is the alteration in angiogenic proteins such as the Vascular Endothelial Growth Factor (VEGF) and the Platelet Derived Growth Factor (PDGF). These are essential for cancer migration, differentiation and induction of mitosis (Reviewed by Rosen *et al*. 2010).

C. Mechanotransduction

1. Overview

Mechanotransduction covers the cellular process that converts mechanical signals into biochemical ones, consequently assisting cells in their adaption to their physical neighborhood by sustaining an appropriate structure and function for a proper tissue homeostasis. It even plays a role in stem cell differentiation during development based on the extracellular matrix surrounding the cells. This is important to guide the cells to their exact fate and exact location. Not only does mechanotransducion play a role in

development, it is a part of the regulation of many processes in the body such as the hypertrophic growth (increase in size) of muscle tissue in response to physical exercise (Reviewed by Jaalouk*et al*. 2009).

2. Mechanotransduction Alterations and Subsequent Implications

Since mechanotransduction plays an important role in many pathways in the body be it at the developmental level or physiological level, alterations may cause major problems. For example, muscular dystrophy, a muscular disease that hampers locomotion by weakening skeletal muscles, is caused by a mutation in themechanosensoryproteindystrophin responsible in connecting different muscular elements (Reviewed by Wallace *et al*. 2008). Another example is the Hutchinson Gilford ProgeriaSyndrome, a laminopathycharacterized by premature ageing due to defective nuclear mechanics and mechanotransduction (Reviewed by Liu *et al*.2008).

3. Mechanotransduction in Cancer and Breast Cancer

Mechanotransduction is a significant factor because of its direct effect on the ECM. It has been shown that alterations in the cytoskeleton, resulting from altered physical interaction with the ECM are involved in promoting cancer motility and cell growth (Reviewed by Ingber, 2008). On the other hand, tissue rigidity is also a characteristic of

cancer caused by ECM stiffness that activates mitogenic signaling through ERK as well as contractility to increase ECM stiffness (Reviewed by Huang *et al*. 2005).

It is known that breast cancer tissue is stiffer than normal mammary epithelial tissue. Moreover, it was shown that a rigid microenvironment in the breast regulates gene expression and even metastasis (Scott *et al*. 2011). For example a stiff ECM activates mitotic genes and other pathways leading to a stiffer ECM, increasing proliferation, deregulation of cell polarity and disorientation (Reviewed by Jaalouk*et al*. 2009). Moreover, alteration in mechanosensitive genes such as *paxillin* and *hic-5* also lead to tumor progression because of their location at the focal adhesion complex that could easily alter signals from the outside of the cell to the inside and could affect their migration and invasion potential. Their alteration also leads to epithelial to mesenchymal transition (EMT) (Deakin*et al*. 2012). In this latter study, it was shown thatcells depleted of paxillin exhibited an elongated mesenchymal morphology, whereas Hic-5 knockdown stimulated an amoeboid phenotype leading to reduced plasticity and migration persistence in a 3D context.

4. Mechanosensitive Genes

1. TWIST1

a. Overview

The *TWIST* 1 gene is located on the human chromosome 7p21. It encodes for the protein TWIST 1 that is a helix loop helix (two α-helices connected by a loop) transcription

factor (reviewed by Aniseau*et al*. 2010). It can have activating or repressing functions depending on the gene downstream (Yu *et al*. 2013) and can bind and regulate the function of other transcription factors by forming homo or heterodimers (Castanon*et al*. 2001). Research has shown that *TWIST1* is necessary for development; twist 1 null mice die at embryonic day 10.5 presenting numerous defects such as abnormal limb buds and closure failure of the neural tube (reviewed by Aniseau*et al*. 2010). In adult tissues, TWIST 1 is responsible for maintaining the undifferentiated state of many precursor cells namely chondroblasts, osteoblasts, and the myogenic lineage (Pan *et al*. 2009). At the level of the immune system, it is induced after B cells stimulation, and is necessary for the memory B cell proliferation, differentiation and survival. On the other hand, it is implicated in the prevention of pro-inflammatory response of the lymphocytes and macrophages by inhibiting the production of many cytokines such as INF α and TNF-α respectively (Niesner*et al*. 2008), (Sharif *et al*. 2006).

b. Role in Cancer and Breast Cancer

TWIST 1 expression is highly induced in many carcinomas including breast, lung, kidney, and prostate among others in addition to sarcomas and melanomas where it is associated with metastatic phenotype (Puisieux*et al*. 2006; Ansieau*et al*. 2008). Moreover, *TWIST 1* is located in an unstable chromosomal region which may subject it to chromosomal rearrangements hence, amplification in cancer cells which is the case for colon and cervical cancer for example (Aragane*et al*. 2001), (Choi *et al*. 2007). Additionally, TWIST 1 is implicated in EMT, a process that takes part in metastasis, by up-regulating the mesenchymal markers N-cadherin and vimentinand down regulating

epithelial markers such as E-cadherin (Onder*et al*. 2008). TWIST 1 is related to cancer phenotype by preventing apoptosisthroughthe inhibition of the tumor suppressor p53 and retinoblastoma (pRb) (Ansieau*et al*. 2008).Moreover, the mechanostransduction function of TWIST 1 is confirmed by being an essential mechano-mediator involved in the promotion of EMT in response to increased matrix stiffness. High matrix stiffness promotes the release of Twist 1 from its cytoplasmic binding partner (G3BP2) and its subsequent translocation to the nucleus (Wei S. 2014).

The mammary tissue is also prone to cancer initiation and progression mediated by TWIST1alteration. Research has shown that *TWIST 1* is highly expressed in the stroma of mammary carcinoma but is very rare in the epithelial compartment in the same cancer type (Soini*et al*. 2011). This indicates its implication in the metastatic phenotype of this carcinoma via EMT because it would be in favor of the mesenchymal phenotypes that is crucial for metastasis. More interestingly, it assists in metastasis to the bones through miR10b, a pro-invasive factorwhich leads to bone destruction (Croset*et al*. 2014).

2. Cathepsin D

a. Overview

Cathepsins are lysosomalproteolytic enzymes (mostly endopeptidases) with a wide range of physiological and pathological states such as bone remodeling, maturation of the MHC class II complex,apoptosis,keratinocyte differentiation, tumor progression and metastasis(Reinheckel*et al*. 2007). They are comprised of three subtypes which are the serine proteases (cathepsins A and G), aspartic proteases (cathepsin D and E), and the

cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, X and W) (Conus*et al*. 2008), and are synthesized as non-functional proteins directed to the lysosome to get activated (Ishidoh*et al*. 2002). The aspartic proteinase cathepsin D (CTSD) has many target proteins, and contributes in many ways to the normal function of the cell. For example, it is responsible for the degradation and activation of many hormones, growth factors and their receptors along with the degradation of many ECM proteins. It is also crucial for the activation of many precursor enzymes, and is involved in apoptosis and LDL cholesterol hydrolysis (Benes *et al*. 2008). In addition, Cathepsin D is regulated by mechanical forces. Research on osteoarthritis has shown that loading *in vivo*, and high intensity exercises modulated*CTSD* expression whereas the expression of other cathepsins such as cathepsinB was not affected (Bowe *et al*. 2007).

b. Role in Cancer and Breast Cancer

Given that Cathepsin D has many crucial functions in the cell, it is very likely that any mutation or alteration in it will lead to diseases. Indeed, this is the case in cancer. It has been reported that cancer cells have high levels of *CTSD* and that it acts as an autocrine growth factor for many types of cancers including lung and prostate. Precursor Cathepsin D is also involved in cancer invasion and metastasis, since studies have shown that as its level increases, metastasis potential increases as well (reviewed by Tan *et al*. 2013).
Likewise, in breast cancer, Cathepsin D is implicated in cancer progression and metastasis in that it acts as anautocrine growth factor, and it also attenuates the anti-tumor immune response by digesting many chemokines expressed in mammary carcinoma (Wolf *et al.*2003). Moreover, *CTSD*isshown to be differentially expressed in the two types of

mammary carcinoma since its expression is elevated in invasive ductal carcinoma compared to the lobular type (Dian *et al*. 2014).

3. Caveolin1

a. Overview

*CAV1*is located on chromosome 7 and encodes for the protein caveolin-1 (Cav-1), a membrane protein crucial for the formation of caveolae (small invaginations of the plasma membrane involved in endocytosis, signal transduction among many others). In invertebrates, the caveolingene family is comprised of the three members *Cav-1, Cav-2, and Cav-3*. *Cav-1 and Cav-2*are co-expressed in many cell types whereas *Cav-3* is restricted to muscle cells (Reviewed by Rahman*et al*. 2009). Cav-1 has mainly a transmembrane domain, an –oligomerizationdomain including a caveolin scaffolding domain and a C terminal membrane attachment domain. *Cav-1* gives rise to two isoforms; α and β. The difference between these two isoforms is the deletion of 31 amino acid residues at the N-terminus of the β isoform due to either alternative splicing or initiation. This deletion results in a 21kDa Cav-1β comprised of 148 amino acids versus a 24 kDa Cav-1α that consists of 178 amino acids (Boscher and Nabi, 2012). Another consequence of this deletion in the β isoform is the absence of a tyrosine phosphorylation site that is present in the α isoform at residue 14. The phosphorylation process leads to the accumulation of Cav-1 at the focal adhesion sites and the transduction of various intracellular signals. (Hehlgans and Cordes. 2011). Cav-1 plays a role during development as its absence is implicated in ductal hyperplasia (Lee *et al.* 2002). Moreover, caveolin is considered to be a mechanosensor because of its ability,for example, to detect the chronic exposure to shear

stress by increasing the caveolin proteins on the plasma membrane (Rizzo *et al*. 2003) that would subsequently induce many signaling pathways(Radel*et al*. 2005).

b. Role in Cancer and Breast Cancer

Regarding cancer progression and development, Cav-1 has a paradoxical role because of its dualcontext-dependent implication in tumor suppression and in oncogenesis. Thedownregulation of Cav-1 is implicated in the progression of ovarianand colon cancer while its up-regulation is linked to squamous cell carcinoma of the esophagus, pancreas, prostateand lung cancer(reviewed by Hehlgans and Cordes, 2011).

Caveolin-1 is associated with many tumor suppressors. For example in the human breast adenocarcinoma cell lineMCF7, ectopic expression of Cav-1 resulted in an up-regulation of BRCA1 through a p53 dependent mechanism (Glait*et al*. 2006). Similarly, caveolin-1 was shown to regulate the phosphatase and tensin homolog protein (PTEN) which possesses tumor suppressor functions (Caselli*et al*. 2002). By contrast, the oncogenic property of caveolin-1 has been shown in many *in vitro* and *in vivo* experiments. A study of 930 breast cancer patients reported an overexpression of caveolin-1 in metastatic or deceased patients (Garcia *et al*. 2007).

4. Heat Shock Protein 70

`a. Overview

The 70 KDa heat shock protein (HSP70) belongs to a family of proteins with housekeepingproperties.All the HSP 70 homologs are comprised mainly of an ATPase domain and a substrate binding domain. The ATP state has a low affinity with a fast

exchange rate for substrate whereas the ADP state has the opposite patterns (Mayer and Bukau, 2005). HSP70 is responsible for the folding and assembly of new proteins and refolding of damaged or misfolded proteins. Accordingly, it is commonly referred to as the 'buffering' protein because of its ability to maintain the normal function of a stress-induced mutated protein by using the abovementioned methods. Stress can be caused in the cells by many factors, including oxidative stress, high temperatures, altered pH and hypoxia. If HSP70 is overwhelmed with protein repair, the risk of disease increases because of the increase in pathological mutant proteins such as the case of Parkinson for which the cause is mutated α-synuclein.

HSP70 is implicated in apoptosis inhibition by acting on many caspase-dependent and independent pathways. Moreover, its overproduction is responsible for resistance against the anti-neoplastic agent doxorubicin, staurosporin and other apoptosis-inducing agents. On the other hand, decreased HSP70 levels lead to enhanced sensitivity towards these agents. Furthermore, Hsp70 is a mechanosensor; stress-induced HSP70 production is a general mechanism by which these proteins provide protection to sensory cells. For example, Hsp70 is induced by heat shock from multiple therapeutic drugs by glias cells in the ear to provide protection against death for the sensory hair cells. Any impairment or death in these sensory cells can lead to hearing and balance problems. Hsp70 plays a role in avoiding damage to specific cells by mechanotransduction (May L.*et al*. 2013).

b. Role in Cancer and Breast Cancer

Hsp70 is implicated in cancer progression. Itis reported to have elevated expression in many tumors where it is associated with increased malignancy. For instance, increased

expression of Hsp70 has been found to be associated with vascular invasion in gastric cancers. Overexpression of HSP70 member 1 for example confers a tumorigenic phenotype to murine fibrosarcoma and makes them resistant to killing by many immune cells such as macrophages and cytotoxic T cells (Jäättelä 2005). Moreover, it was suggested that Hsp70 is involved in cancer cell adhesion and metastasis since its depletion leads to cell detachment. Knockdown of Hsp70 in cervical and bladder cancer was shown to reduce invasion and migration (Juhasz. *et al*. 2014).

Moreover HSP70 overexpression is implicated in breast cancer progression. Silencing HSP70 with antisense RNA provoked significant cell death in breast cancer cells while the normal breast epithelial cells were not affected. Elevated expression of Hsp70 was found to correlate with lymph node metastasis in breast cancer cells(reviewed by Juhasz.*et al*. 2014).

E. Cancer Treatments

1. Overview

Cancer treatments are very broadand diverse due to the multitude of cancer types and cancer causes. As such, there are multiple therapeutic approaches that could be used for many cases. One of the most commonly used therapeutic approach for cancer is surgery in that it has a high cure percentage and could be used for prevention. Chemotherapy and radiation therapy are also widely used to kill cancer cells; the former is based on the utility of drugs that interfere with proliferating cells and the latter uses high energy particles to destroy cancer cells. A more recent modality for cancer treatment which was shown to be

very effective is targeted therapy whereby the drug is targeted to the cancerous tissue while sparing normal tissues. Immunotherapy is also used to aid the immune cells towards the cancerous cells in order to fight cancer. Moreover, extreme temperatures were shown to be effective when it comes to cancer therapy, for example using heat to treat cancer is called hyperthermia and cryosurgery is the use of intense cold produced by liquid nitrogen to demolish selected abnormal cells (American Cancer Society, 2014).

2. High Intensity Focused Ultrasound (HIFU)

a. Overview

High Intensity Focused Ultrasound (HIFU) is a therapeutic device widely used in Eastern Asia and Europe to destroy unwanted tissues inside the body. It was first introduced by Lynn *et al*. in the 1940's as a non-invasive(ex-corporal) and non-ionizingmodality for the treatment of neurological disorders. Multiple improvements have been made to HIFU technology since then and it is now being used for the treatments of many types of cancer such as prostate, liver, pancreatic, breast cancer and more. Its importance lies in the fact that it is used where surgery and radiation cannot be of use (Yunbo*et al.* 2005; Zhang *et al.* 2010).

A HIFU transducer has an advantage over the normal ultrasound transducer because of its concave shape that intensifies the energy at the focal spot, whereas the latter one dissipates the energy (Figure 1).

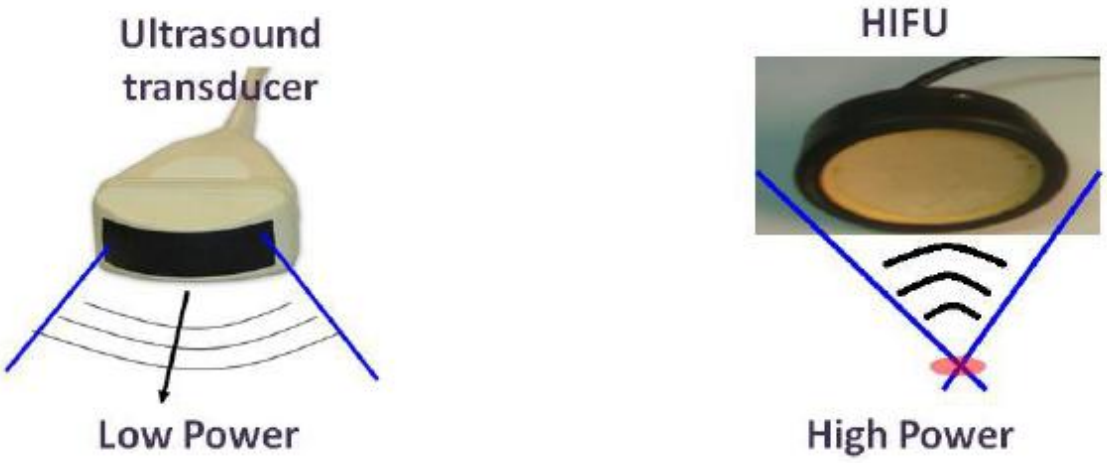


Figure 1: An illustration showing the regular ultrasound transducer and the HIFU transducer (Malkhassian L., MSc Thesis 2012).

HIFU works at low and high energy with different resultant effects. At low intensity, slow heating takes place with instantaneous raise in temperature above normal levels (Prasanna*et al.* 2008). On the other hand, when HIFU is operated at high energy, bubbles form due to a rapid alteration in pressure; these bubbles will then expand and burst damaging nearby cells(Khokhlova*et al.* 2008). This phenomenon known as cavitation mainly happens at the focal spot, that is 1-3mm long and <1mm in cross section,where the acoustic waves from a HIFU transducer are maximally intensified (TerHaar*et al.* 2007). Moreover, this intensified energy leads to an instantaneous (few milliseconds) rise in temperature in the tissue (which may reach 60°C) causing necrosis to the cells subjected to HIFU at the focal point.However, cells in the surrounding tissue that receive residual energy are thought to be left unharmed (Reviewed by Rove *et al.*2010).

One of the advantages of HIFU application for tumor ablation over the use ofstandard therapies is the potential of pain relief by ablating the coeliac ganglion responsible for pain in any abdominal cancer treatment without injuring the spinal cord or adjacent aorta (Foley *et al.* 2007).

b. Biomedical Applications of HIFU

The biomedical applications of low power HIFU include fracture repair, gene therapy, and physiotherapy among others; while at high power, both the thermal and mechanical effects of HIFU allow its use in multiple therapeutic applications. Aside from its use in ablation fo solid tumors, HIFU could be used to treat vascular occlusion (TerHaar, 2007) and in the fragmentation of kidney stones (Yoshizawa*et al.* 2009).

c. HIFU Treatment

i. In Cancer

Coupled to improved imaging techniquesthat allow direct and real-time observation during treatment, the use of HIFU to treat numerous types of cancers has grown in recent years. These cancers include renal cell carcinoma, primary hepatocellular carcinoma (HCC), secondary metastasis, thyroid, parathyroid tumors, breast cancer, bone tumors and uterine fibroids, (Kennedy *et al*. 2004); (Orsi*et al.* 2010). However, the most common HIFU - treated type of cancer is prostate cancer because of its very satisfactory outcome where almost all the gland was successfully ablated in prostate cancer (Reviewed by Takeo *et al.* 2012).

In Breast Cancer

The use of HIFU for breast cancer ablation is becoming more and more common mainly because it has no effect on the breast structure and function, it can be applied in successive rounds of treatment, and does not cause bleeding (Li and Wu, 2013). In clinical trials, it was shown by a study conducted by Wu *et al*. that complete breast tumor ablation using HIFU is feasible with no leftover clumps (Wu *et al*. 2005.). Moreover, a phase II HIFU treatment done in Japan reported only one case of recurrence out of 21 cases after almost one year of observation (Furusawa*et al.* 2007). These studies provided strong support for the use of HIFU as a treatment option for breast cancer patients who do not desire surgery. Nevertheless, long-term observations are necessary to avoid any recurrence that could lead to metastasis (Hwang. *et al.* 2009).

3. Anti-neoplastic Agents

Cancer chemotherapy is based on the administration of certain chemicals with the purpose of destroying tumor cells. In1940, the first successful chemotherapy treatment was reported in lymphomas. It came with the incidental discovery that theexposure to mustard gas (a weapon used during World War II) resulted in the death of white blood cells in exposed individuals (reviewed by Nygren, 2001).

Chemotherapy agents, also referred to as anti-neoplastic agents, vary tremendously in how they work. For instance, anti-metabolites act mainly at the level of the DNA in

thatthey get incorporated in the DNA sequence because of their analogous structure to the nucleotide bases. This way, the integrity of the sequence will be affected because it will be depleted of its original base units leading to an arrest of DNA replication or in some cases to fatal defects in its structure (Lansiaux*et al*. 2011). Alkylating agentsact directly on DNA and lead to cross-linking, abnormal base pairing, mutation (alkylated guanine bases might incorrectly pair with thymine), DNA strand breaking, and eventually cell division inhibition and cell death. The repair system can also be affected since mono-alkylation obstructs the DNA enzymes from accessing the DNA sequence resulting in apoptosis and cell growth arrest(Ralhanet and Kaur,2007). Topoisomerase inhibitors, as their name indicates, inhibit the work of the topoisomerases. Under normal conditions these enzymes are responsible for the cleavage of the DNA strands to allow successful replication. The topoisomerase inhibitors therefore lead to an interruption of DNA replication hence cell death (Pilati*et al*. 2012).

a. Classification And Types Of Chemotherapy Agents

i. Paclitaxel

- Overview

Mitotic inhibitors are a class of anti-neoplastic agents, but their method of action is quite different from the abovementioned ones. They inhibit mitosis by several mechanisms and they include the microtubulin enzyme inhibitors, mitosis enzyme inhibitors, mitosis checkpoint inhibitors, and the microtubulin binders to which Paclitaxel, also known as Taxol, belongs (review Tsao and Papadimitrakopoulou, 2011*).* Used initially for ovarian cancer treatment in 1992 (Sarosy*et al*. 1992),Taxol's isolation from the stem bark (phloem

and cambium) of *Taxusbrevifolia*dates back to 1971(Wani*et al*. 1971). Later on, it was harvested in the Pacific Northwest (Cragg*et al*. 1991). It is the first active constituent of *Taxusbreuifolia* extract to be used in clinical trials because of its significant cytotoxic effectson many tumors (Wani*et al*. 1971).

Under normal conditions, tubulin heterodimers (composed of alpha and beta subunits) are involved in many processes such as maintenance of cell shape, motility, intracellular trafficking along with the formation of the mitotic spindle (Winey and Bloom 2012).

- Mechanism of Action

Taxol acts mainly at the microtubule level, promoting their polymerization unlike other microtubule drugs that act on their disassembly. More specifically, it binds next to the M loop in the beta monomer causing it to stabilize the lateral interaction between proto-filaments (Orr *et al*. 2003; Nogales 2000). This phenomenon, allows the microtubule filaments to become more stable and more resistant to depolymerization by cold, and calcium ions (Figure 2).Taxol- treated cells show a deregulation in the G2/M checkpoint and it is suggested that this is due to the inability of these cells to form a healthy mitotic spindle or to dissociate Taxol from the microtubule (Zhang. *et al*. 2014).

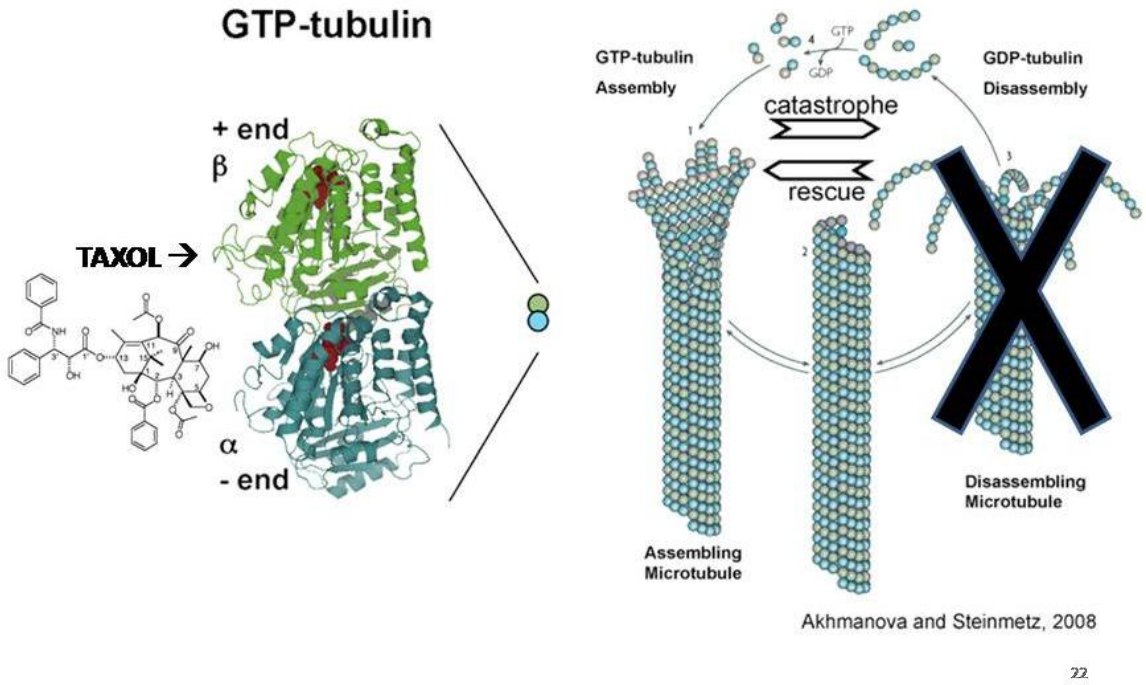


Figure 2: An illustration of Taxol's mode of action; upon binding to tubulin, it enhances the interaction between proto-filaments in such a way that it stabilizes the assembly of microtubules and inhibits the disassembly (amended from pnas.org and Akhmanova and Steinmetz 2008).

- Resistance to Paclitaxel

Although Taxol is an effective drug, resistance is also a major concern. Cancer cells take advantage of many processes to resist the drug. It has been hypothesized that resistanceemerges from normal cells to avoid carcinogens in the environment. The possible mechanisms of resistance include altered uptake of the drug through the plasma membrane, altered genetic responses and target molecules, and enhanced DNA repair. The main mechanism of Taxol resistance is its extrusion by the P-glycoprotein (P-gp) which is a 170 kDa protein encoded by the multidrug resistant (MDR1) gene. Moreover, studies on breast cancer showed a possible involvement of the ECM in resistance in thatthe ligation of β1

integrins by the ECM inhibited Taxol- mediated apoptosis (Reviewed by Luqmani 2005). In addition, it has been shown that Taxol-resistant cell lines have “hypostable” microtubules that shift more to dimer equilibrium (Munoz Fontena*et al.* 2008). As such, Taxol and other polymer-binding drugs activity will be compromised and attenuated.

ii. Doxorubicin

- Overview

Doxorubicin (Doxil), also known as Adriamycin, is an anthracyclin antibiotic drug produced by the *Streptomyces peucetius* bacteria. It was discovered in 1960 near the Adriatic Sea, hence the name (Blum et al. 1974).It has been shown to be effective against many cancer types includingleukemia, breast, ovarian and lung cancer among many others (Duggan and Keating. 2011).

- Mechanism of Action

Doxorubicin acts at the level of the DNA by inhibiting replication of proliferating cells. It binds to the DNA by various means such asthe formation of strands by a hydrogen bond between the hydroxyl group of doxorubicin and the N7 position of either guanine or adenine (Jae Lee *et al*. 2004; Perez-Arnaiz*et al*. 2014). This intercalation alters the DNA structure leading to inhibition of the enzymes involved in DNA transcription and replication. Of the many enzymes inhibited is topoisomrase2 which is responsible for the

unbinding of the supercoiled DNA. When the drug is intercalated onto the DNA strand, the function of the topoisomerase 2 in rejoining the unbound strands is hindered leading to apoptosis by the activation of some caspases (Sordet*et al.* 2003).

DNA intercalation also inhibits DNA Methyltransferase 1 (DNMT1) activity (Figure 3);thus leading to deregulation of gene expression followed by apoptosis in human colon cancer cells (Yokochi and Robertson, 2004). Moreover, it forms iron-mediated free radicals that cause oxidative damage of proteins, nucleic acid and membrane lipid (Barenholzet*et al.*2012).

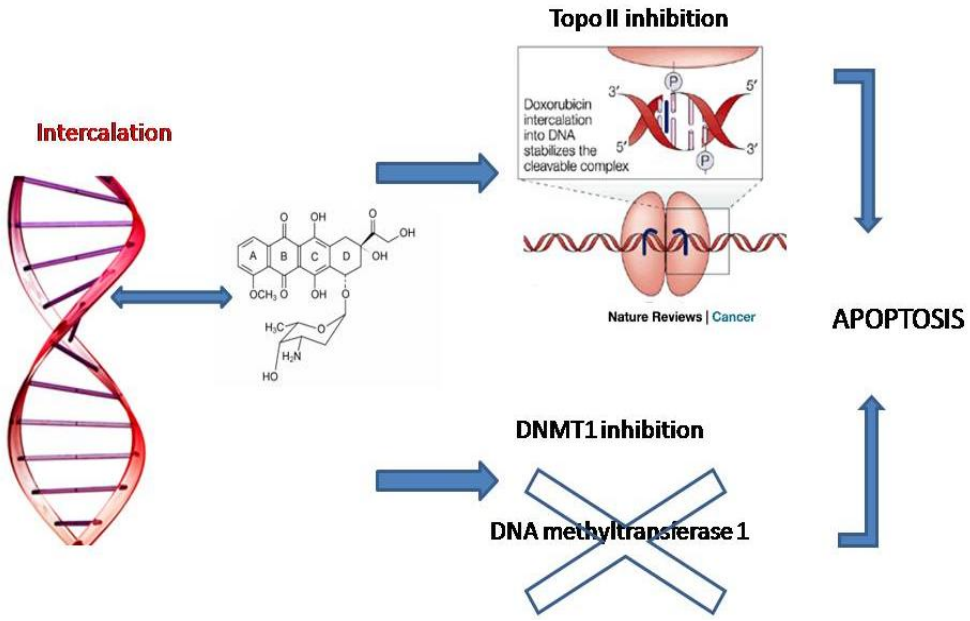


Figure 3: A schematic illustration of Doxorubicin's mode of action. Doxorubicin intercalates in the DNA chain and inhibits topoisomerase II as well as DNA Methyl Transferase. The outcome is apoptosis (amended from scienceandbelief.org, Nature review, and Sang Lee *et al*. 2004).

- Resistance to Doxorubicin

The resistance to doxorubicin resembles in part the mechanism involved in the resistance to Taxol where the MDR1 P-gp glycoprotein is involved. It is shown that

doxorubicin is extruded by these proteins from the cell, hence conferring resistance(Reviewed by lukmani, 2005). A specific example of doxorubicin resistance is the substitution of arginine at position 283 with either glycine or threonine in the ABCG2/BCRP which is a "half-type" ABC transporter of 72 kDa. Moreover, a variant of this protein with substitution at position 482 also confers resistance to many chemotherapy drugs including doxorubicin in murine fibroblast (Noguchi *et al*. 2014). Additionally, caveolin-1 has also been implicated in conferring resistance against doxorubicin (Raghazan and Shajahan, 2014) attributed to the increase in MDR playing a role on the membrane assisting in evacuating the drug from the treated cells.

4. Combinatorial Therapy

1. Overview

Combinatorial therapy aims at providing the best remedy for the patient. It involves mainly adjuvant and neo-adjuvant therapy, where the former is any therapy given after the main or primary treatment to increase long-term survival and the latter is the administration of therapy prior to the primary treatment. Regarding breast cancer for example, the most common adjuvant therapy includes hormonal therapy, chemotherapy, or radiation therapy (Lancet 2005). For the case of HIFU, research has shown that the combination of chemotherapy and HIFU on prostate cancer bearing rats has a synergistic effect by slowing tumor growth (Paparel*et al*. 2004).

F. Gap in Knowledge, Rationale, and Hypothesis

Many types of solid cancers are good candidates for HIFU ablation. Prostate cancer, for instance, is an ideal candidate for such therapy because of the potential for near whole ablation of this organ if need be (Takeo *et al*. 2012). In the case of pancreatic cancer, HIFU was successfully used asa treatment option for relieving pain. Other cancers treated with HIFU include liver, parathyroid, bone and breast cancer (Orsi*et al*. 2010). The advantage of using intensified ultrasound application is mainly to spare the patient from having to be subjected to an invasive procedure (Tanter*et al*. 2007). Ideally, HIFU would serve as an alternative treatment option for patients not interested in having a surgical operation. A Phase II HIFU clinical study that was performed on 21 breast cancer patients reported positive outcomes with only one case of cancer recurrence noted at 14 months post HIFU application reported(Furusawa*et al*. 2007). However, the authors stated that long-term observation is necessary to monitor any resistance or recurrence later on. Moreover, MCF7 breast cancer cells exposed to HIFU showed an inhibition in proliferation post treatment hypothetically due to abnormal DNA synthesis with no significant change in pro-apoptotic markers such as BCL-1 and p53 (Wang *et al*. 2004). Another recent paper demonstrated that HIFU force could be used to help in chemotherapeutic agent targeting using the nano-particle technology by helping these agents migrate to the middle of the tumor (Oh *et al*. 2014).

Sub-lethal HIFU treatmentby the pulsed HIFU model(Al Qraini*et al*. 2012) might provoke alterations in cancer cells because of the pressure/tension waves that could cause cellular deformations in exposed tissue due to putative alterations in

mechanotransduction(Paszek*et al.* 2005; Jaalouk *et al*. 2009; Al Qraini*et al*. 2012; Malkhassian L MSc Thesis 2012). Furthermore, mechanotransduction alterations and changes in the expression of multiplemechanosensitive genes have been implicated in breast cancer tumorigenesis (Baker *et al*. 2010; Whelan*et al.* 2010; Scott *et al*. 2011). With reference to the above, **we hereby hypothesizethat sub-lethal HIFU treatment results in mechanotransduction alterations that may induce tumorigenesis of mammary epithelial cells and may modulate their response to anti-neoplastic agents.**

G. Objective of the Study and Specific Aims

The broad objective of this study is to examine the alterations in mechanotransduction and the response to anti-neoplastic agents due to changes in physical properties resulting from the exposure of breast cancer cells and "normal" immortalized mammary epithelial cells to ultrasonic waves from a HIFU device; more precisely where some cells receive residual exposure (presumably sub-lethal). The proposed study is comprised of two main specific aims:

Aim 1: To assess the *in vitro* effects of sub-lethal HIFU exposure on mechanosensitive gene expression in the mammary epithelial cell lines MCF-10A and MDA-MB-231.

This aim is a continuation of the work of a former MSc student in our laboratory, whereby four mechanosensitive genes were tested to assess if any alteration in their expression levels in the two cell lines is observed in response to sub-lethal HIFU exposure.

In this study, we aim to test four more genes: i) *CAV1* gene encoding for both caveolin-1 α and β isoforms, *ii) TWIST 1* gene that encodes for Twist-Related Protein 1, iii) *CTSD* gene encoding for Cathepsin D protein, andiv) *HSPA1A* gene that encodes for Heat Shock 70 kDa protein.

Expression levels will be quantified and normalized to that of the reference gene *GAPDH* encoding for the glyceraldehyde 3-phosphate dehydrogen serving as a control between independent experiments. The transcriptional levels of the selected mechanosensitive genes normalized to those of *GAPDH* will be quantified in the MDA-MB-231 and MCF-10A cell lines under the following experimental settings; cells that were subjected to a variety of HIFU parameters versus mock controls, followed by RNA extraction at 1hr and 24hr post HIFU application, then Real Time PCR for gene quantification.

Aim 2: To test the *in vitro* effects of sub-lethal HIFU exposure on the cytotoxic response of MCF-10A and MDA-MB-231 cells to anti-neoplastic agents namely Doxil and Taxol. This aim is further divided to two sub-aims depending on the mode of HIFU exposure:2.1 To determine cellular viability inthe two cell lines in response to Doxil orTaxol treatmentfollowing a single round ofsub-lethal (sub-cavitation) HIFU exposure;amd2.2 To determine cellular viability in the MDA-MB-231 cell line in response to Doxil orTaxol treatment following either a single or a dual round of HIFU exposure at the focal point.

For aim 2.1, two suboptimal cytotoxicconcentrations for Taxol and Doxorubicin will be tested following sub-lethal HIFU exposure to determine if there is a synergistic or antagonistic effect between the two therapeutic applications. Accordingly, cells subjected to sub-lethal HIFU exposure will be seeded at a density of 20, 000 cells per well in a 96 - well plate. Taxol or Doxil at suboptimal cytotoxic concentrations will then be added at 6hror 30hr following HIFU treatment. These time points were selected to account for the timespan needed to account for putative changes in immediate - early (30min – 6hr) or delayed expression (24-30hr) of select mechanosensitive genes that may be altered following sub-lethal HIFU exposure and may modulate cellular response to anti-neoplastic agents. Subsequently, cellular viability was determined using the trypanblue stain exclusion assay that was performed on days 1-to-4 following treatment with Taxol or Doxil.

For aim 2.2, cells exposed to HIFU at the focal point; i.e. receiving 100% of the applied energy, yet survive the application will be passaged and maintained in tissue culturefor a period of 3 weeks. Afterwards, cells will be seeded and treated with suboptimal cytotoxic concentrations of Taxol or Doxil as stated above for aim 2.1. The time of chemotherapy addition, however, was one day post seeding.

CHAPTER II

MATERIALS AND METHODS

A. Maintenance of Cells in Tissue Culture

1. MCF-10A

MCF-10A is a non-tumorigenic immortalized mammary epithelial cell line that was established from a fibrocystic lump of human mammary epithelial cells. The cell linewas kindly provided by Dr. RabihTalhouk's laboratory, Department of Biology, AUB. MCF-10A cells are cultured using Dulbecco's Modified Eagle's Medium Ham's F-12 (DMEM-F12) media (Lonza) supplemented with 1% penicillin-streptomycin.Two types of media preparations were used for this cell line; one of which is the re-suspension media (80% DMEMF-12 with Pen-Strep and horse serum (Lonza), and the second preparation is the growth media that is supplemented to the re-suspension media with insulin(Sigma), cholera toxin (Enzo),hydrocortisone (Sigma), and human epithelial growth factor (Peprotech Inc.).The doubling time of this cell line is about 29hr. At 80% density confluence, cells are washed with 1× Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO-Invitrogen) then with 2×Trypsin (Lonza) to detach them from the plate. They are then incubated for 40-45 min in a 37°C,5% CO_2incubator. Re-suspension media is then added to neutralize the effect of trypsin and the cells are centrifuged using 600g at 4°C for 5min. Afterwards, an adequate

amount is added to the cell pellet then distributed in 10cc plates pre-filled with 10ml growth media.

2. MDA-MB-231

MDA-MB-231 is a human breast adenocarcinoma cell line with a highly invasive phenotype. It was also provided by Dr. RabihTalhouk's laboratory. These cells are cultured using RPMI 1640 media (Sigma-Ibra Haddad) to which Pen-Strep and 10% Fetal Bovine Serum (Sigma-Aldrich) are added. The protocol followed for this cell line is similar to the one used for MCF-10A cells, but the trypsinizationtime is about 30sec. The doubling time for the cells is about 26hr.

B. HIFU Experiments

1. Cell Preparation Pre-HIFU

MCF-10A or MDA-MB-231 at 80% confluence are trypsinized and centrifuged as previously mentioned, then distributed over 0.2ml PCR tubes with a density of 2×10^5 cells in each tube. After preparation, the tubes are placed in a thermostatic container and transported to the Department of Mechanical Engineering at AUB, where the HIFU setup (Figure 4) is located. The sample tubes are immersed in the reservoir/HIFU tank which contains degassed, distilled H_2O pre-heated to 37°Cand they are locked in place for HIFU application using adjustable holders/clamps. The various HIFU exposures are appliedbyadjusting the position of the clamps close to or away from the transducer such that: i) cells exposed at the focal point (FP; 44mm from the transducer)are expected to

receive 100% of the energy from the intensified acoustic waves, ii) cells exposed at varying distances of 42.5mm, 43.5mm, and 43.6mm from the transducer are expected to receive residualenergyfrom the propagated acoustic waves estimated at 25.7%, 75.7%, and 83% respectively of that received at the focal pointbased on the consideration that the intensity distribution of the HIFU field follows the Gaussian beam profile (Curra FP *et al*. 2000; Soneson JE *et al.* 2007),and iii) cells immersed in the tank without being subjected to any exposure (mock control). Residual HIFU exposure is presumably sub-lethal since sub-cavitation HIFU is believed to leave cells in nearby tissue unaffected. The different experimental parameters used are as follows: Volts: 256Vpp, frequency: 2Hz, temperature of water tank: 37°C, duty cycle: 2%, sonication time: 10msec, and total exposure time: 200msec.

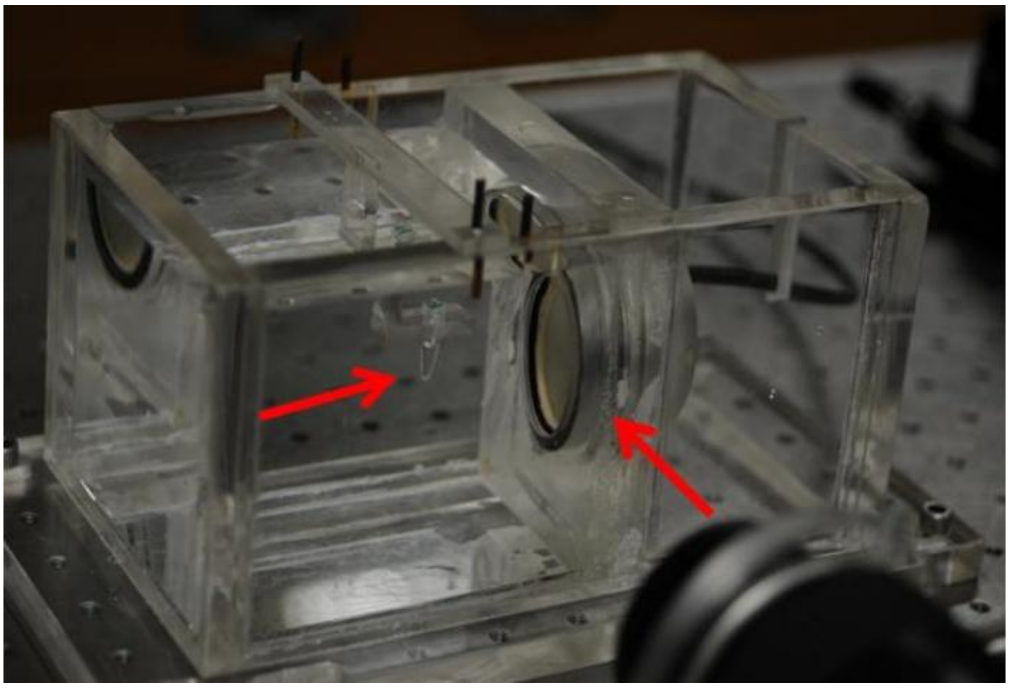


Figure 4: A photographic image of the HIFU cell-suspension setup (Courtesy of the laboratory of Dr. GhanemOweis, Department of Mechanical Engineering, FEA, AUB).

2. Cell Re-suspension and Seeding Post HIFU

Post HIFU exposure, the MDA-MB-231 and MCF-10A cells are diluted and re-suspended with growth media, then seeded at a density of $2x10^4$/100µl cells in a 96-well plate, then placed in an incubator. These cells are cultured for 4 time points, once per 24hr increments. For the cells that were subjected at the focal point, very few of them survive. That is why they need to be continuously maintained in a 6-well plate. As they reach adequate density, they are then transferred to a 10cc plate for passaging over 3 weeks prior to performing Taxol or Doxil treatment assays.

C. Quantification of Gene Expression

1.RNA Extraction

RNA extraction from MCF10-A and MDA-MB-231 was performed by a former student in the laboratory (Malkhassian L MSc Thesis, 2012). The extraction was done at different time points post HIFU exposure. The first time point aims at assessing the response of potentially early-activated genes post sub-lethal HIFU for which RNA extraction was done 1hr post exposure. The second time point aims at assessing potentially delayed gene expression (24hr post exposure). In the 1hr post-HIFU application time point, RNA was directly extracted from cells situated inthe sample tubes by RNeasy Kit (QIAGEN) according to the manufacturer's protocol. Similarly, RNA was also extracted from the mock control group. For the 24hr time point post HIFU application, RNA was extracted

from cells seeded in 6-well plates. At the time, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and the extraction method was performed similarly as for the 1hr time point. The quantity and quality of the extracted RNA samples were assessed by Nanodrop Spectrophotometer, and then all the RNA samples were stored at -80°C(Malkhassian L MSc Thesis, 2012).

2. Reverse Transcription

cDNA reverse transcription was done on RNA from each sample using theiScriptcDNA Synthesis Kit (Bio-Rad) and following the manufacturer's protocol specifications. In brief, 1µg of RNA was mixed with 1µl of the reverse transcriptase enzyme, 4µl of the Reaction Mix, and 16µl of nuclease free sterile water in a total volume of 20µl. The reverse transcription protocol consists of 3 steps; 5min at 2°C, 30min at 42°C, followed by 5min at 85°C. The samples were then stored at -20°C(Malkhassian L MSc Thesis, 2012).

3. Real-Time PCR

The Real-Time PCR amplification was performed using theiQ SYBR Green Supermix (Bio-Rad) and specific primer pairs for each selected gene. The primer sequences for the Real-Time PCR amplification of theselected genes were computationally derived from the MGH/Harvard Medical School Primer Bank Database (Table 1).The reverse transcribed cDNA is diluted to a 1:20 ratio (5µl cDNA with 95µl nuclease free sterile water). Then 5µl of nuclease free sterile water, 1µl of reverse and forward primer (for each gene) and 12.5µl mastermix are added to 4µl of the diluted cDNA derived from the different samples in a

total volume of 25µl. The reaction is performed using a Real-Time PCR machine (Bio-Rad) following this protocol: Heating step for DNA denaturation starting with 50°C, then increased to 95°C for 10min. Subsequently, the temperature is lowered to 60°C for 1min to allow the primers to anneal to their complementary strands after which the temperature is shifted to 72°C for 30sec. This cycle is repeated 40 times. The final step is performed at the same temperature, but for 10min. All experimental results were analyzed by the BIO-Rad CFX Manager Software.

Table 1: A list of the forward and reverse primers used to quantify select mechanosensitive genes by Real-Time PCR.

Gene	**Primer**	**Sequence 5′-3′**
Cav-1(α and β)	Forward	CATCCCGATGGCACTCATCTG
	Reverse	TGCACTGAATCTCAATCTCAATCAGGAAG
HSPA12A	Forward	GCTCCCACATCTGCATATTCAT
	Reverse	TTCTGAGACGTTGGAGTCAGT
TWIST1	Forward	GTCCGCAGTCTTACGAGGAG
	Reverse	GCTTGAGGGTCTGAATCTTGCT
CTSD	Forward	TGCTCAAGAACTACATGGACGC
	Reverse	GTCCCGATGCCAATCTCCC
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAAC
	Reverse	GGGGTCATTGATGGCAACAATA

D.Anti-neoplastic Agents Preparation and Addition

A specific concentration of each of the two anti-neoplastic agents (Paclitaxel and Doxorubicin) is prepared then added to the cells either at 6hr or 30hr following HIFU exposure. Dilutions are prepared for each drug to obtain the desired working concentrations; Paclitaxelis diluted usingdimethyl sulfoxide (DMSO) whereas water is used to dilute Doxorubicin. The chosen concentrations for Paclitaxel are 1.5nM and 7.5nM and the ones for Doxorubicin are 0.05μM and 0.5μM (Results section; figures 5 and 6).

E. Viability Assays

1. Microscopic Observation (Phase-Contrast)

Cells, post HIFU exposure and pre-treatment with Taxol or Doxil, as well as post drug treatment, but prior to determining viable cell counts were monitored by microscopic observation using a standard phase contrast microscope with a 40x magnification to observe cell shape and morphology, cell density, and to rule out potential experimental artifacts or contamination.

2. Trypan Blue Vital Stain Exclusion Assay

At 24hr post treatment with Taxol or Doxil, MCF-10A and MDA-MB-231 cells pre-seeded in 96-well plates are washed with Phosphate Buffered Saline (PBS) 1× then trypsinized,

and neutralized with the appropriate re-suspension media. They are then diluted with Trypan Blue on a 1:1ratio of which 10µl is transferred to a hemacytometer counter grid for viable cell count using a light microscope. The same procedure is done over 4 days.

F. Statistical Analysis

The statistical analysis was done using one way ANOVA. The calculations were achieved using SPSS program. A p-value of 0.05 represents the statistical significance. Furthermore, the average of each group, the standard deviation and the standard error of the mean were calculated and a comparison of each group to their control group was done. Unless stated otherwise, all experiments were performed in duplicates three independent times.

CHAPTER III

RESULTS

A. Parameter Optimization in the Methods Used

1. Optimization of HIFU Parameters to Ensure Mechanical Sub-lethal Exposure

The parameters for the HIFU set up that we have been usingin the laboratory had to be re-optimized because the setup that was used in previous studies was disassembled and the transducer was sent for calibration in the UK. Following calibration and re-assembly, it was necessary to re-check the parameters and to validate if exposures similar to those used previously will result in similar effects on cell viability in the two tested cell lines. Secondly, different users were in charge of operating the transducer so small adjustments needed to be made to ensure accuracy and reproducibility. The modifications made were primarily related to the focal point; we needed to re-determine the exact focal point location so that we could identify by calculations the distances from the transducer that would result in sub-lethal exposure. Moreover, the adjustable clamps used to hold the sample tubes also had to be changed from Plexiglas to aluminum since the latter ones hold more tightly in place and make it easier to achieve reproducibility.

2. Optimization of Seeding Density of Cells PostHIFU

The seeding density of cells in the 96-well plates had to be tested inorder to avoid too high or too low confluence pre-and post-drug treatment for 4 days. We started with a $5x10^3$cells/well density (based on published literature), but differentsuboptimal cytotoxic concentrations of both Taxol and Doxilhad robust and invariant cytotoxic effects on both cell lines. We attributed this result to the very low cell density that was initially used since even minimal cell death that would result from treatment with suboptimal cytotoxic concentrations of either drug would compromise the ability of the remaining viable cells to pick up due to very low cell density. So we increased the cell density to $20x10^3$cells/well and were successful in getting variant cytotoxic effects when cells were treated with increased suboptimal cytotoxic doses of either drug.

3. Determining the Suboptimal Cytotoxic Concentrations of Taxol and Doxil

We tested various concentrations of Taxol and Doxorubicin on the MDA-MB-231 and MCF-10A cells to determine those that would result in suboptimal cytotoxic effects (i.e. less than optimal or complete effect on cell viability). The rationale for using suboptimal cytotoxic concentrations of either drug is to enable us to determine if sub-lethal HIFU treatment would modulate cellular response to these agents by acting in synergy with chemo (sensitizing cells to Taxol or Doxil) or by being antagonistic (de-sensitizing cells to Taxol or Doxil). After performing several trials testing both cell lines with varying doses of either drug, we selected two concentrations for each: 0.05μM and 0.5μM for Doxorubicin (Figure 5), and 1.5nM and 7.5nM forTaxol (Figure 6).

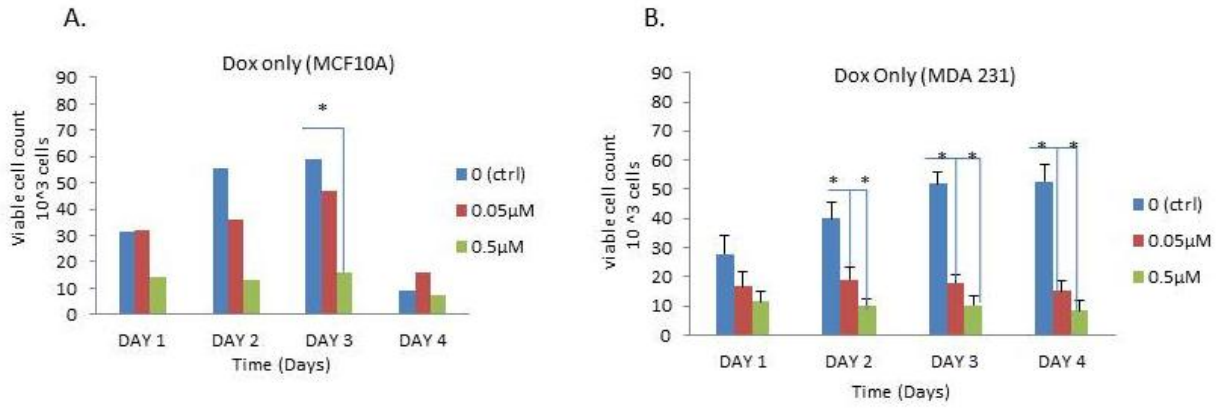


Figure 5: Quantitative assessment of viability of MCF-10A (panel A) and MDA-MB-231 (panel B) cells post treatment with suboptimal cytotoxic concentrations of Doxil.Trypan blue vital stain exclusion assay was used to count viable cells days 1-to-4 following the addition of 0.05µM or 0.5µM Doxil. A significant difference in cellular viability was noted between the control (mock treated) and theDoxil – treated groups in MDA-MB-231 cellsat days 2, 3 and 4, but only at day 3 and with the 0.5µM concentration in MCF-10A cell line. Data represent mean ± SEM and asterisks represent statistical significance ($p<0.05$).

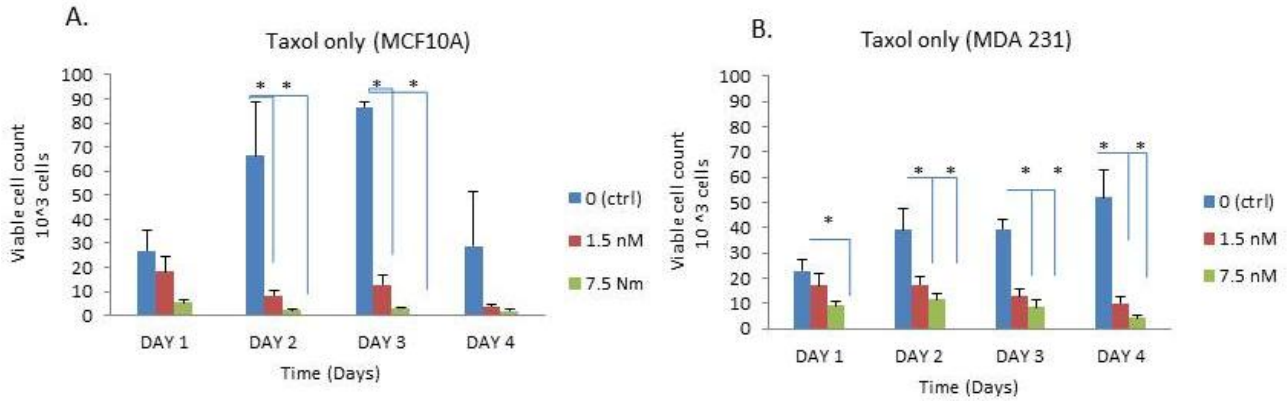


Figure 6:Quantitative assessment of viability of MCF-10A (panel A) and MDA-MB-231 (panel B) cells post treatment with suboptimal cytotoxic concentrations of Taxol.Trypan blue vital stain exclusion assay was used to count viable cells days 1-to-4 following the

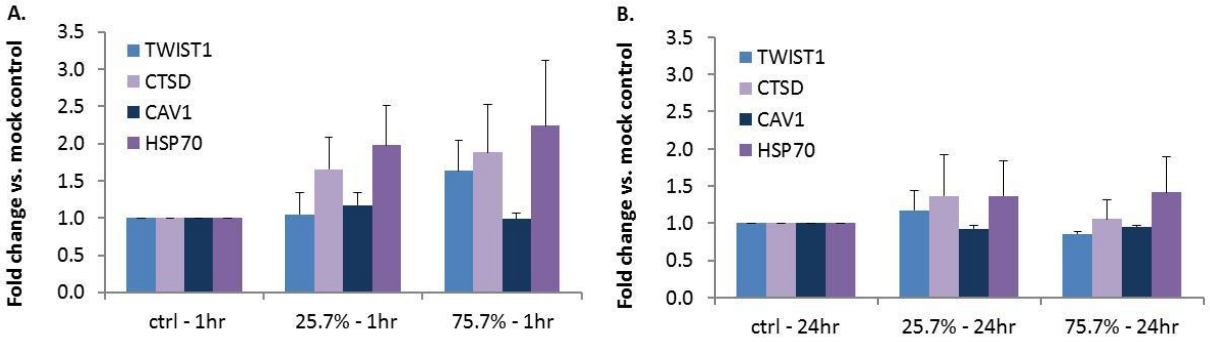
addition of 1.5nM and 7.5nM Taxol. A significant difference in cellular viability was noted between the control (mock treated) and the Taxol – treated groups in MDA-MB-231 cells at days 2-to-4, but only at days 2 and 3 in MCF-10A cell line. Data represent mean ± SEM and asterisks represent statistical significance ($p<0.05$).

B. Modulation in the Expression of Select Mechanosensitive Genes Post Sub-lethal HIFU Exposure

In previous work done by Lori Malkhassian, a former graduate student in the laboratory, we assessed the *in vitro* effects of sub-lethal HIFU exposure on the expression of 4mechanosensitive genes whereby we quantified significant enhanced expression of *CAV-1α*(only the α isoform was tested back then), *PXN*, and *Hic-5* that was immediate-early in MCF-10A cells and delayed in MDA-MB-231 Cells (Malkhassian L MSc Thesis, 2012). Additionally, we noted a significant immediate-early and transient increase in *TTLL4* expression in both cell lines.

Hereby, we continued testing the panel of mechanosensitive genes that we had originally selected by assessing putative changes in the total pool of*CAV-1*(*CAV-1t;* both α and β isoforms), in *TWIST1*, *CTSD*, and *HSPA1A* in both cell lines. Notably, sub-lethal HIFU exposure had no significant effect on the expression of the total pool of *CAV-1*(i.e. combined pool of α and β isoforms) in both cell lines (Figures 7 and 8), hence suggesting that there is a reduction in the expression of the β isoform of *CAV-1* that is immediate-early in MCF-10A cells and delayed in MDA-MB-231 cells (in accordance with the observed changes in the α isoform that were reported previously and mentioned above). Similarly, sub-lethal HIFU exposure had no significant effect on the expression of *CTSD* and *HSPA1A* in both cell lines (Figures 7 and 8). Interestingly, we noted a significant

immediate-early and transient increase in *TWIST1* expression in MDA-MB-231 cells, but



not in MCF-10A cells (Figures 7 and 8).

Figure 7:Real-Time PCR quantification of *TWIST1, CTSD, CAV-1*and *HSP70* gene expression in MCF-10A cells at 1hr (panel A) and at 24hr (panel B) following two sub-lethal HIFU exposures at which cells are estimated to have received 25.7% and 75.7% of the intensified energy at the focal point respectively. Data indicate that there is no significant change in the expression of the 4 genes in all tested groups versus mock treated controls at both time points. Data represent mean ± SEM from three independent repeats, each performed in duplicates.

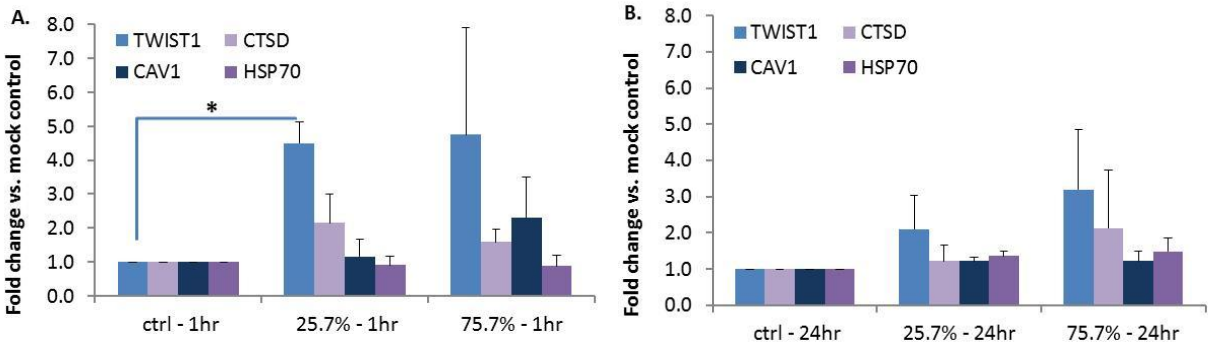


Figure 8:Real-Time PCR quantification of *TWIST1, CTSD, CAV-1*and *HSP70* gene expression in MDA-MB-231 cells at 1hr (panel A) and at 24hr (panel B) following two sub-lethal HIFU exposures at which cells are estimated to have received 25.7% and 75.7% of the intensified energy at the focal point respectively. Data indicate that there is no significant change in the expression of *CTSD, CAV-1*and *HSP70*in all tested groups versus

mock treated controls at both time points. However, there is a significant, but a transient 4.5 (±0.62) -foldinduction in the expression of*TWIST1*at 1hr following sub-lethal HIFU exposure at 25.7% of the focal point intensity. Data represent mean ± SEM from three independent repeats, each performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

C. Sub-lethal HIFU Exposure Sensitizes MDA-MB-231 and MCF-10A Cells to Suboptimal Cytotoxic Doses of Taxol and Doxil

To test the *in vitro* effects of sub-lethal HIFU exposure on the cytotoxic response of MDA-MB-231 and MCF-10A cells to the anti-neoplastic agents Taxol and Doxil, we exposed cells to a 25.7% and 83% sub-lethal HIFU exposures then seeded them in 96-well plates. Subsequently, cells were treated with suboptimal cytotoxic concentrations of Taxol or Doxil at 6hr or 30hr post HIFU application and cellular viability was assessed 24hr later for 4 consecutive days using trypan blue stain exclusion assay.

Overall, the results obtained in this study show that MDA-MB-231 cells are sensitized to suboptimal cytotoxic doses of Taxol (1.5nM, 7.5nM) when the drug was added to cells 6hr(Figure 9) or 30hr (Figure 10) following sub-lethal HIFU exposure; i.e. post HIFU exposure of cells that are situated at a distance from the focal spot which receive residual energy estimated at 26% or 83% of the intensity at that received at the focal pointbased on the consideration that the intensity distribution of the HIFU field follows the Gaussian beam profile (Curra FP *et al*. 2000; Soneson JE *et al*. 2007). Similarly, results obtained in this study indicate that MDA-MB-231 cells are sensitized to suboptimal cytotoxic doses of Doxorubicin (0.05μM, 0.5μM) when the drug was added to cells 6hr (figure 11) or 30hr (Figure 12) following sub-lethal HIFU exposure; i.e. post HIFU exposure of cells that are situated at a distance from the focal spot which receive residual energy estimated at 26% or

83% of the intensity at that received at the focal point. Similar results were obtained with MCF10A cells (Figures 13 – 16).

1. Assessment of MDA-MB-231 Cell Viability Following TaxolTreatment at 6 HoursPost Sub-lethal HIFU Exposure

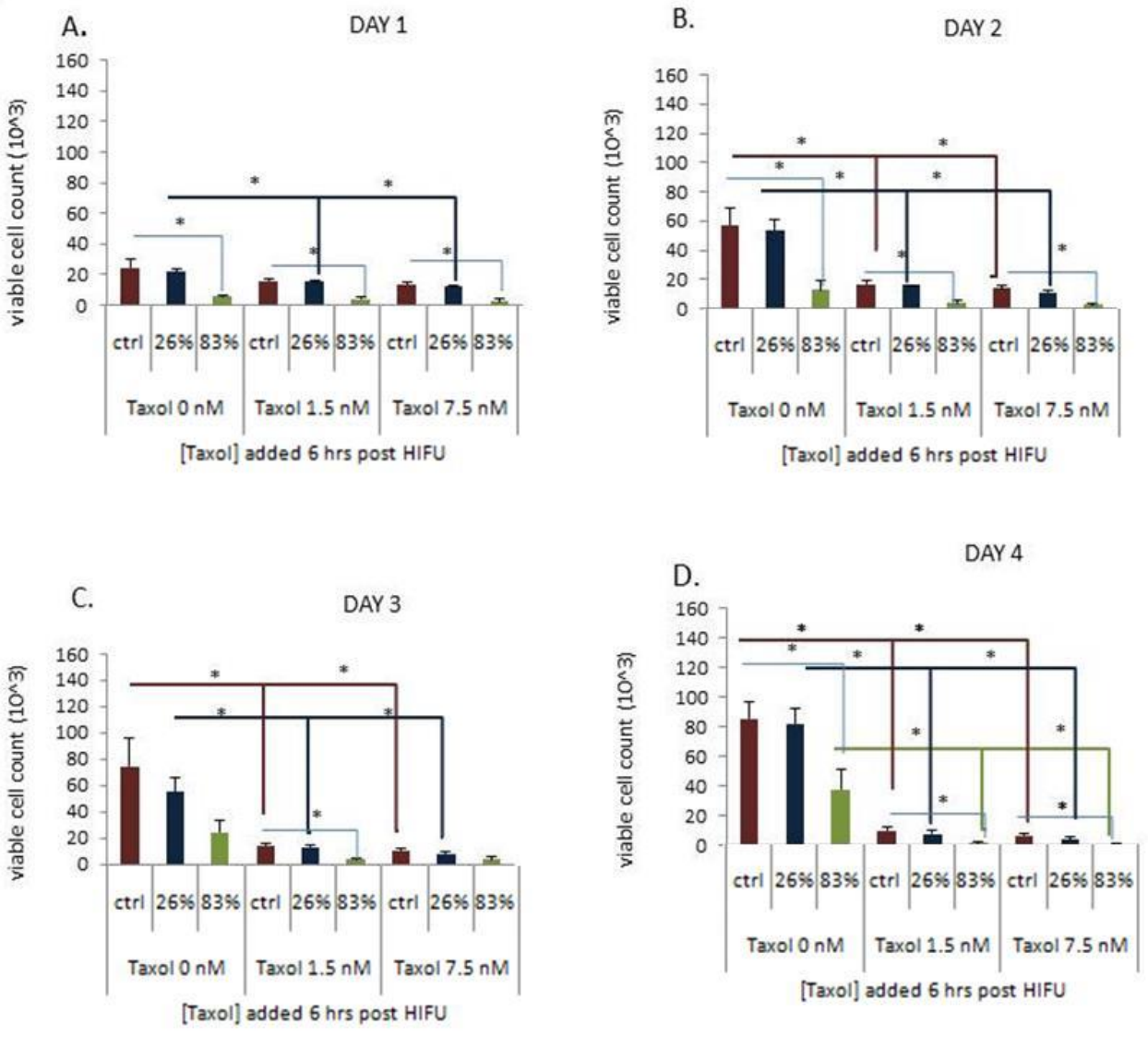


Figure 9: MDA-MB-231 cell viability following Taxol treatment at 6hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over

a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Taxol addition 6 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

2. Assessment of MDA-MB-231 Cell Viability Following Taxol Treatment at 30 Hours Post Sub-lethal HIFU Exposure

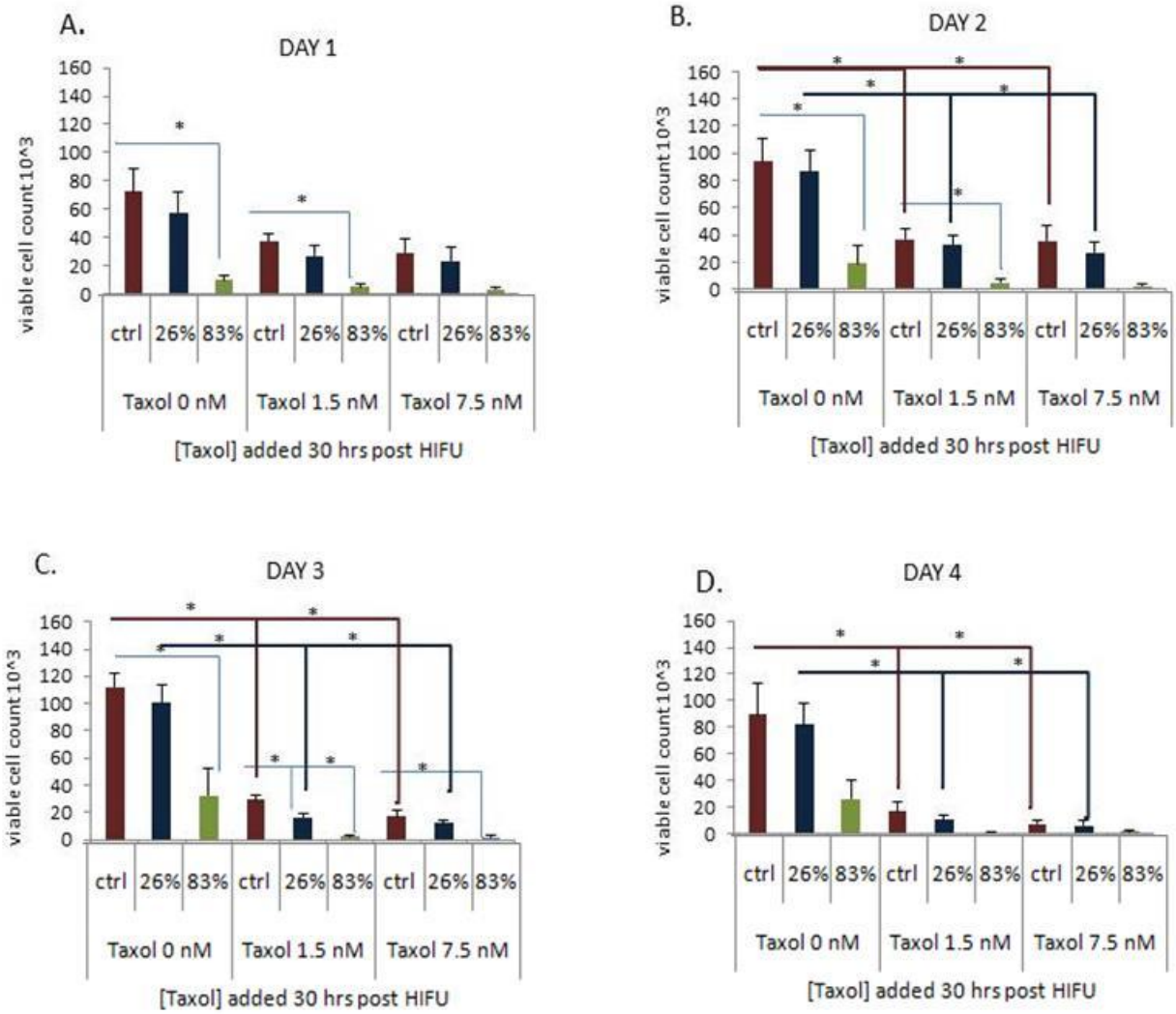


Figure 10:MDA-MB-231 cell viability following Taxol treatment at 30hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over

a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Taxol addition 30 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

3. Assessment of MDA-MB-231 Cell Viability Following Doxil Treatment at 6 Hours Post Sub-Lethal HIFU Exposure

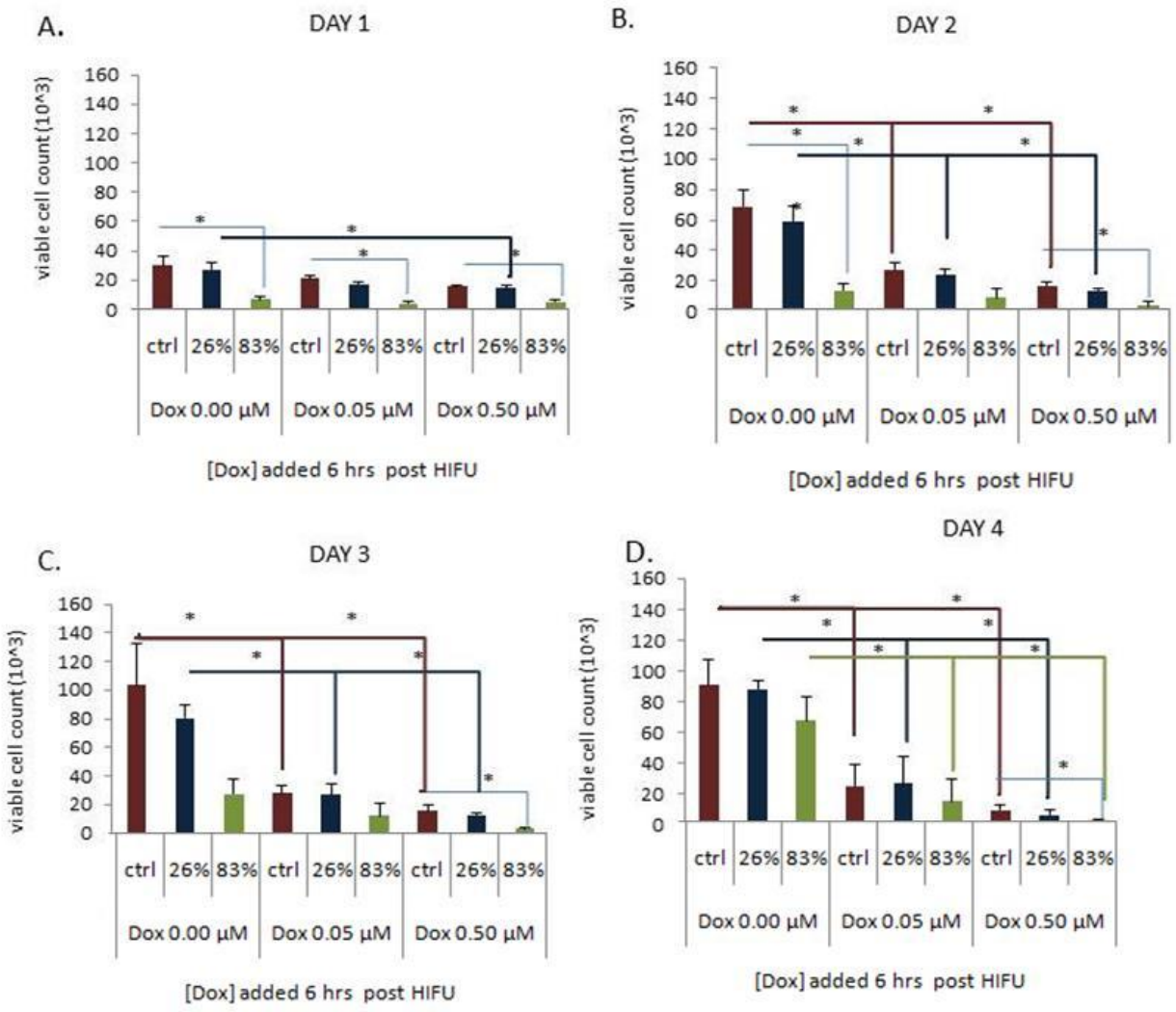


Figure 11:MDA-MB-231 cell viability following Doxil treatment at 6hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%)

followed by Doxorubicin addition 6 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

4. Assessment of MDA-MB-231 Cell Viability Following Doxil Treatment at 30 Hours Post Sub-Lethal HIFU Exposure

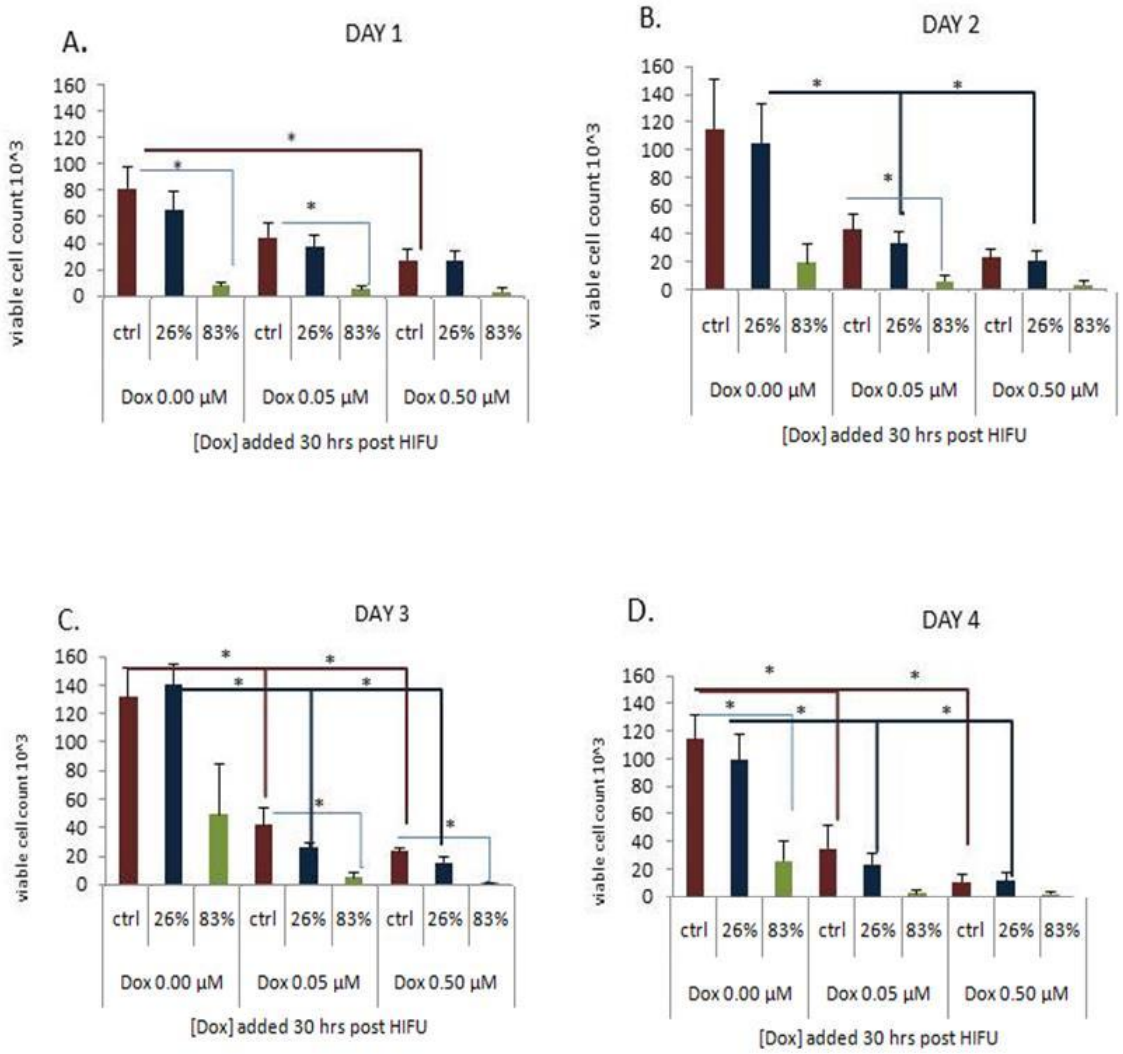
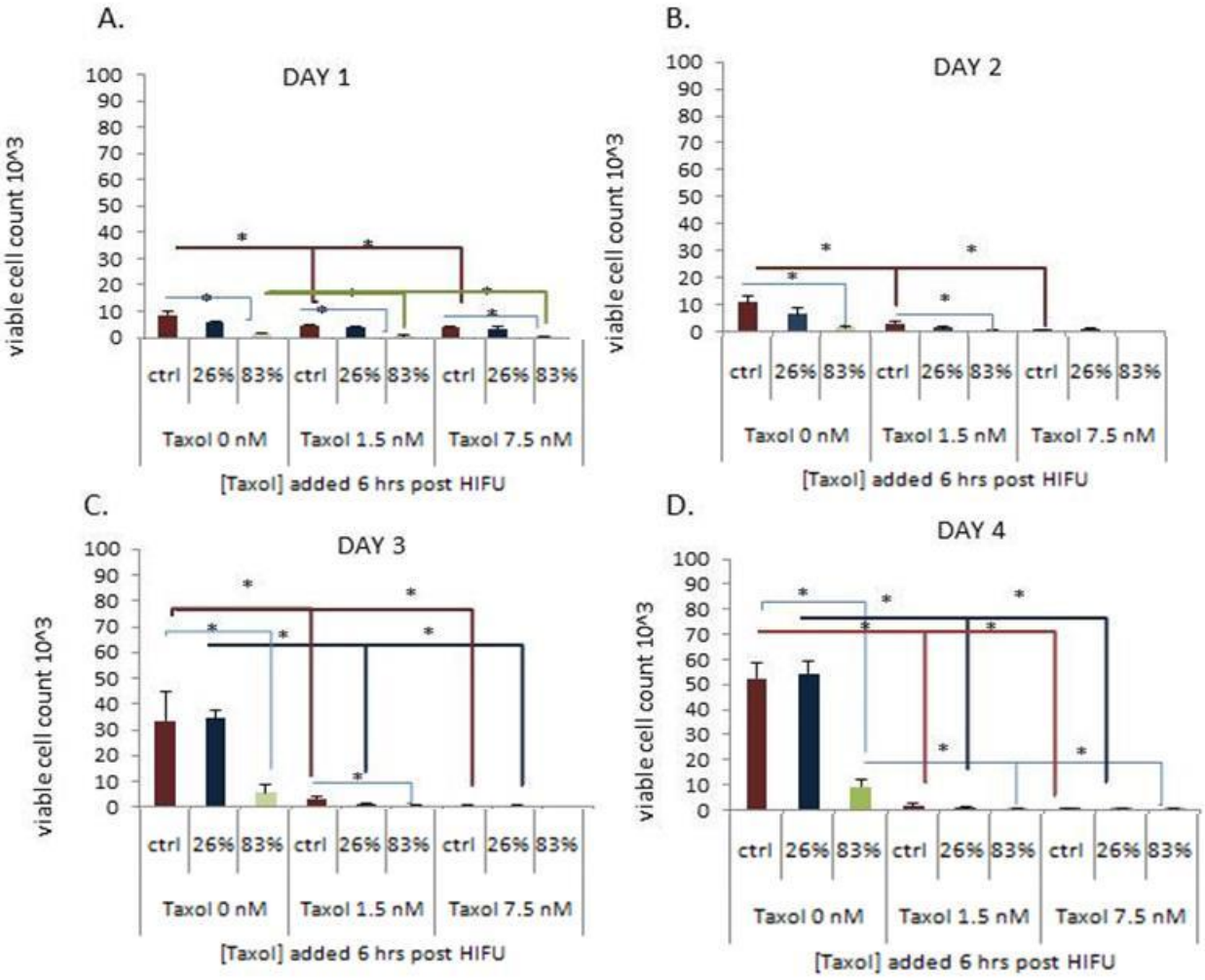


Figure 12:MDA-MB-231 cell viability following Doxil treatment at 30hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Doxorubicin addition 30 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

5. Assessment of MCF-10A Cell Viability Following TaxolTreatment at 6 HoursPost



Sub-lethal HIFU Exposure

Figure 13: MCF-10A cell viability following Taxol treatment at 6hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Taxol addition 6 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

6. Assessment of MCF-10A Cell Viability Following Taxol Treatment at 30 Hours Post Sub-Lethal HIFU Exposure

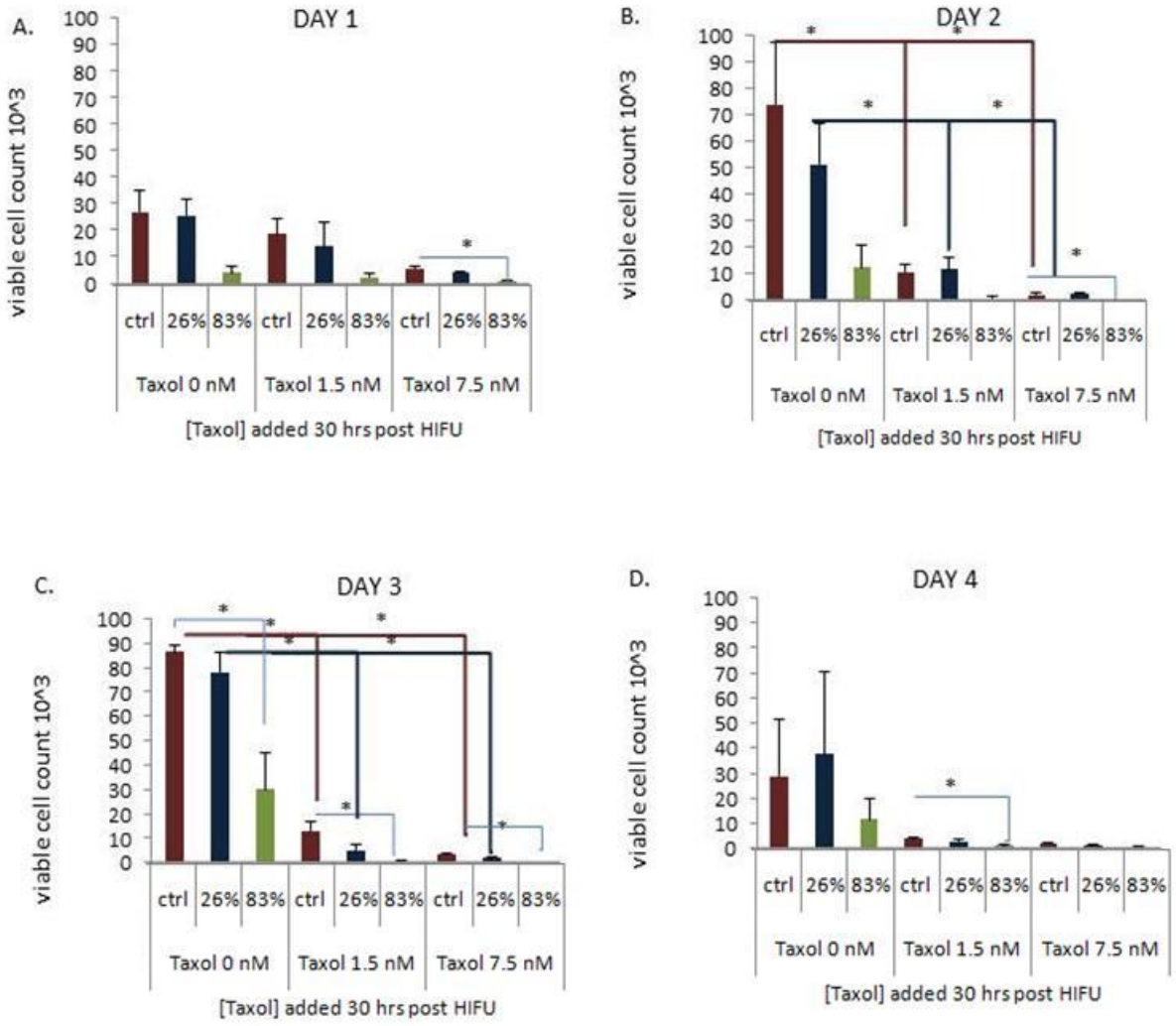


Figure 14:MCF-10A cell viability following Taxol treatment at 30hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Taxol addition 30 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

7. Assessment of MCF-10A Cell Viability Following Doxil Treatment at 6 Hours Post Sub-Lethal HIFU Exposure

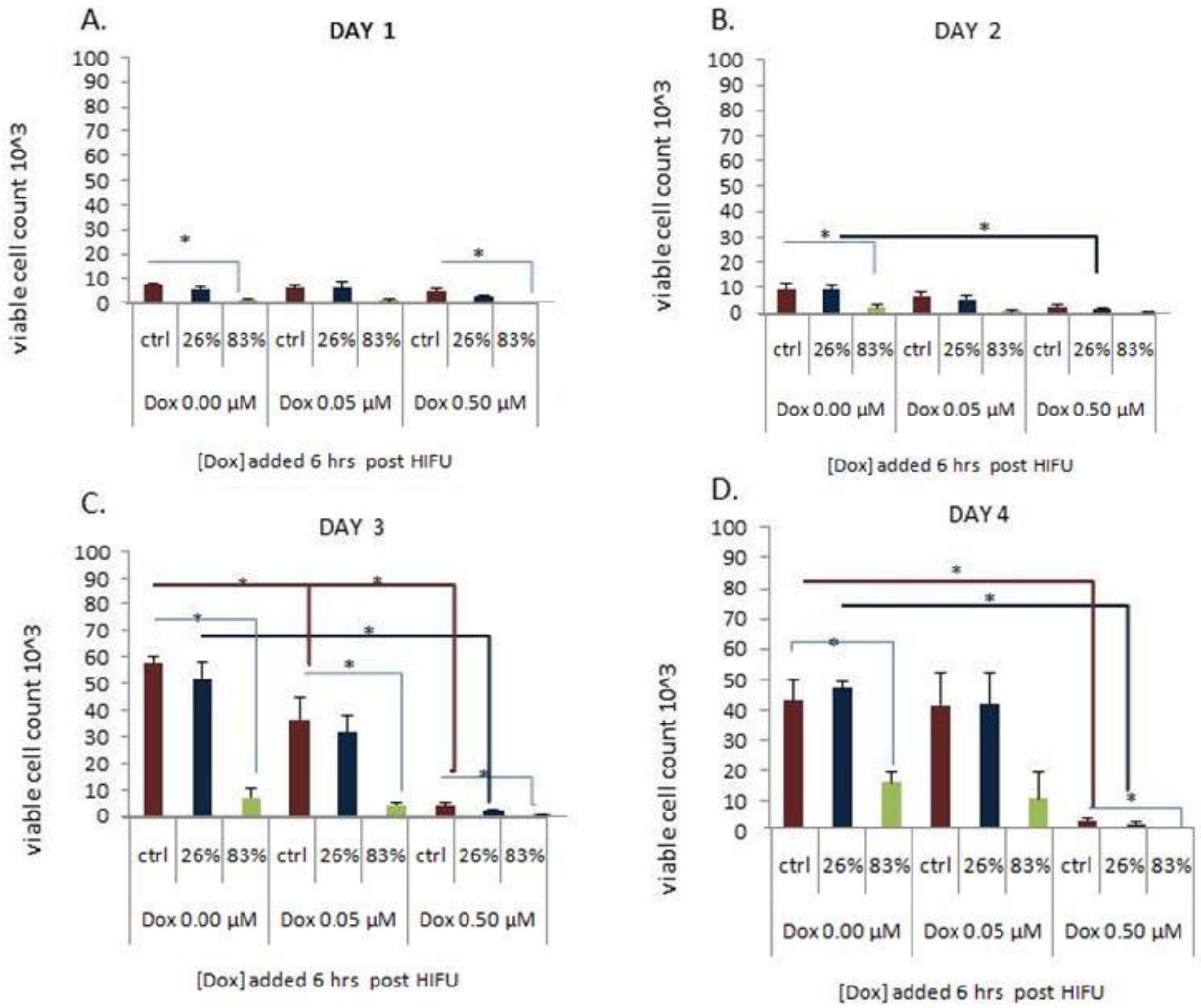


Figure 15:MCF-10A cell viability following Doxil treatment at 6hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Taxol addition 30 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

8. Assessment of MCF0-10A Cell Viability Following Doxil Treatment at 30 Hours Post Sub-Lethal HIFU Exposure

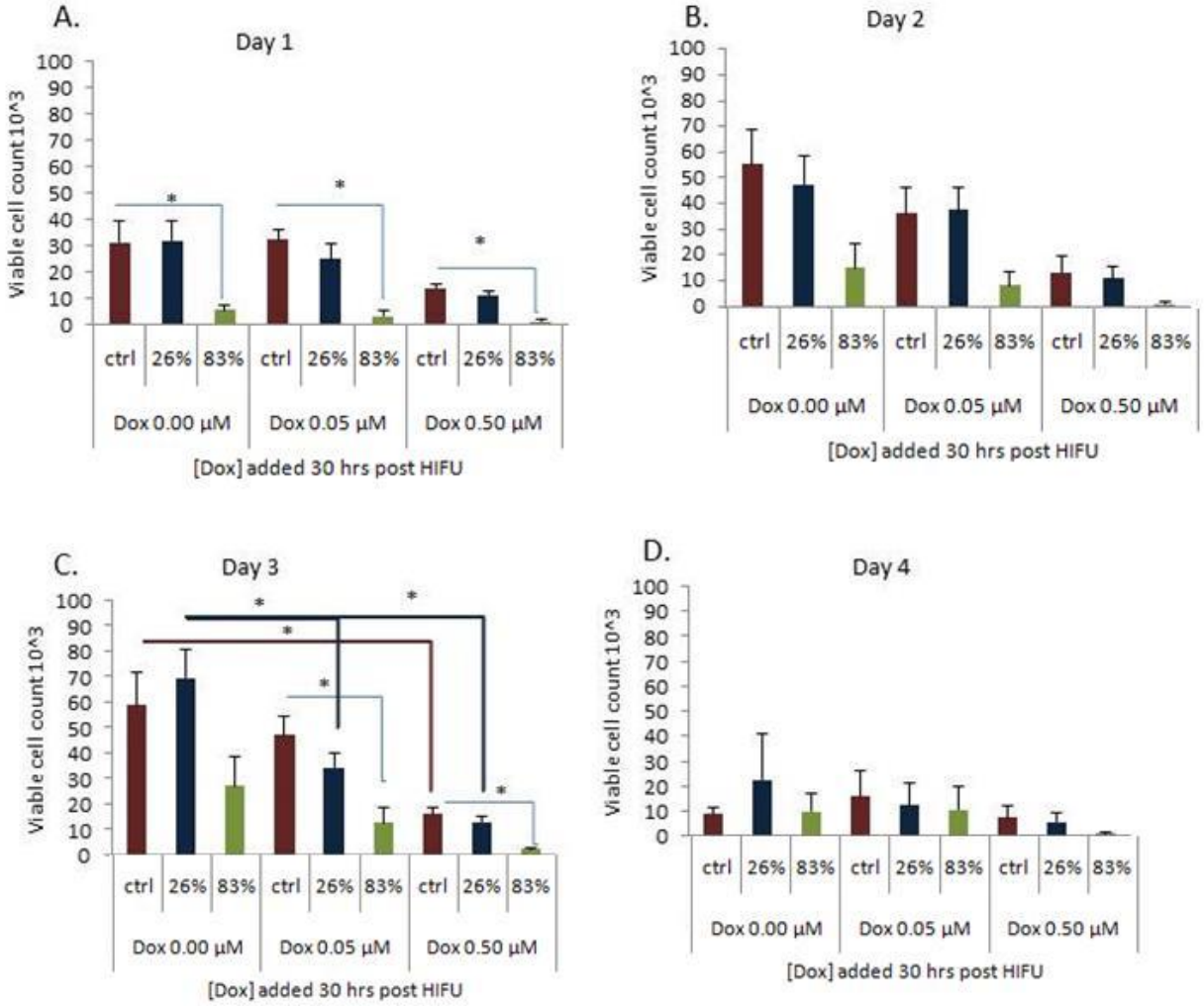


Figure 16:MCF-10A cell viability following Doxil treatment at 30hr post sub-lethal HIFU exposure. Trypan blue vital stain exclusion assay was used to count viable cells over a period of 4 days. Cells were subjected to sub-lethal HIFU exposures (25.7% and 83%) followed by Taxol addition 30 hours post HIFU. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

D. MDA-MB-231 Cells Surviving Single or Dual Rounds of Lethal HIFU Exposure Show No Significant Change in Sensitivity to Taxolor DoxilFollowing Multiple Passages in Tissue Culture

To examine the *in vitro* cytotoxic response to Doxil and Taxol of MDA-MB-231 cells which survive lethal HIFU exposure, we treated cells that survived single or dual rounds of lethal HIFU application at the focal point (100% intensified acoustic energy) and passaged for 3 weeks in tissue culture with suboptimal cytotoxic concentrations of either drug, then determined cell viability after 24hr for 4 consecutive days. The rationale for passaging the cells which survived HIFU exposure at the focal point for 3 weeks is to permit for selection of cells that may potentially have developed resistance toeither one or both chemotherapy agents. It is plausible that cells surviving HIFU at the focal point may be more tolerant to Doxil and/or Taxol. Additionally, they may have acquired *de novo* mutations as a result of cellular deformations that may have resulted from exposure to HIFU at the focal point. Under the experimental conditions tested, our results show that MDA-MB-231 cells surviving single or dual rounds of HIFU exposure at the focal point exhibit no significant change in their sensitivity to Taxol or Doxilfollowing multiple passages in tissue culture in

comparison to mock-treated controls that were passaged and drug tested in parallel(Figures 17 - 20).

1. MDA-MB-231 Cells Surviving a Single Round of HIFU Exposure at the Focal Point Show No Significant Change in Sensitivity to Suboptimal Cytotoxic Doses ofTaxol

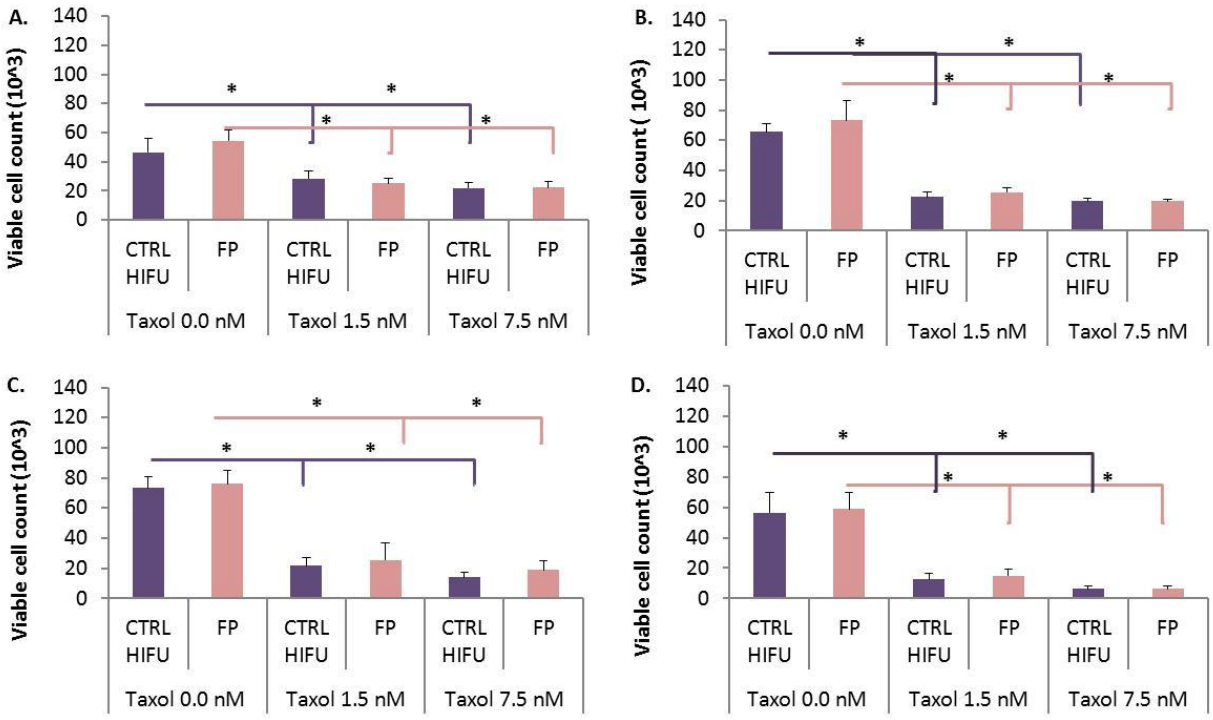


Figure 17:Cytotoxic response to Taxol of MDA-MB-231 cells passaged for 3 weeks in culture post surviving a single round of HIFU exposure at the focal point (FP) in comparison to mockcontrols (CTRL HIFU). Trypan blue vital stain exclusion assay was

used to count viable cells starting with 24hr post Taxol treatment (1.5nM or 7.5nM) for 4 consecutive days (panel A: day 1; panel B: day 2; panel C: day 3; and panel D: day 4). Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

2. MDA-MB-231 Cells Surviving a Single Round of HIFU Exposure at the Focal Point Show No Significant Change in Sensitivity to Suboptimal Cytotoxic Doses ofDoxil

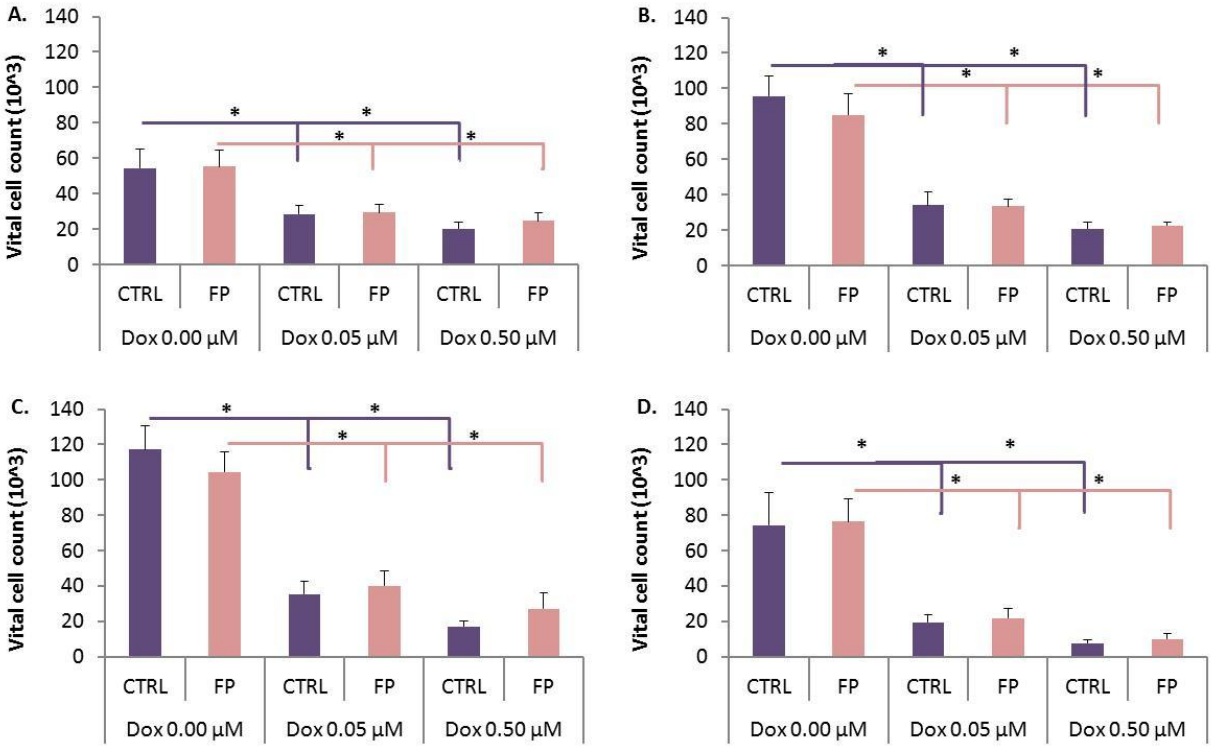


Figure 18: Cytotoxic response to Doxil of MDA-MB-231 cells passaged for 3 weeks in culture post surviving a single round of HIFU exposure at the focal point (FP) in

comparison to mock controls (CTRL HIFU). Trypan blue vital stain exclusion assay was used to count viable cells starting with 24hr post Doxil treatment (0.05μM or 0.50μM) for 4 consecutive days (panel A: day 1; panel B: day 2; panel C: day 3; and panel D: day 4). Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

3. MDA-MB-231 Cells Surviving Dual Rounds of HIFU Exposure at the Focal Point Show No Significant Change in Sensitivity to Suboptimal Cytotoxic Doses ofTaxol

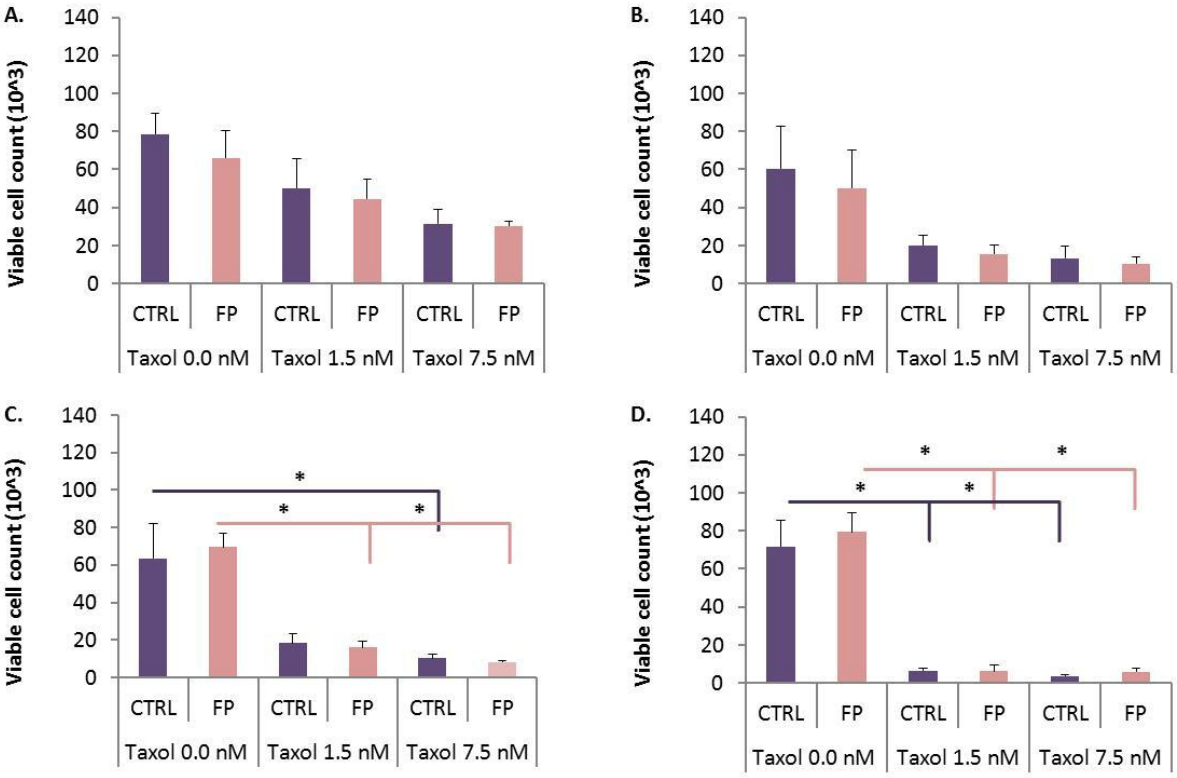


Figure 19: Cytotoxic response to Taxol of MDA-MB-231 cells passaged for 3 weeks in culture post surviving dual rounds of HIFU exposure at the focal point (FP) in comparison

to mock controls (CTRL HIFU). The 2 rounds of HIFU application were performed 3 weeks apart; i.e. cells surviving round 1 were passaged for 3 weeks in tissue culture then subjected to a second round of HIFU. Trypan blue vital stain exclusion assay was used to count viable cells starting with 24hr post Taxol treatment (1.5nM or 7.5nM) for 4 consecutive days (panel A: day 1; panel B: day 2; panel C: day 3; and panel D: day 4). Notably, both mock controls and the cells surviving 2 FP HIFU exposures showed no significant difference in cellular viability in comparison to vehicle treated (0.0nM) Taxol during days 1 and 2 indicating that cells acquired some tolerance to Taxol with advanced passages that was independent of HIFU application. Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

4. MDA-MB-231 Cells Surviving Dual Rounds of HIFU Exposure at the Focal Point Show No Significant Change in Sensitivity to Suboptimal Cytotoxic Doses ofDoxil

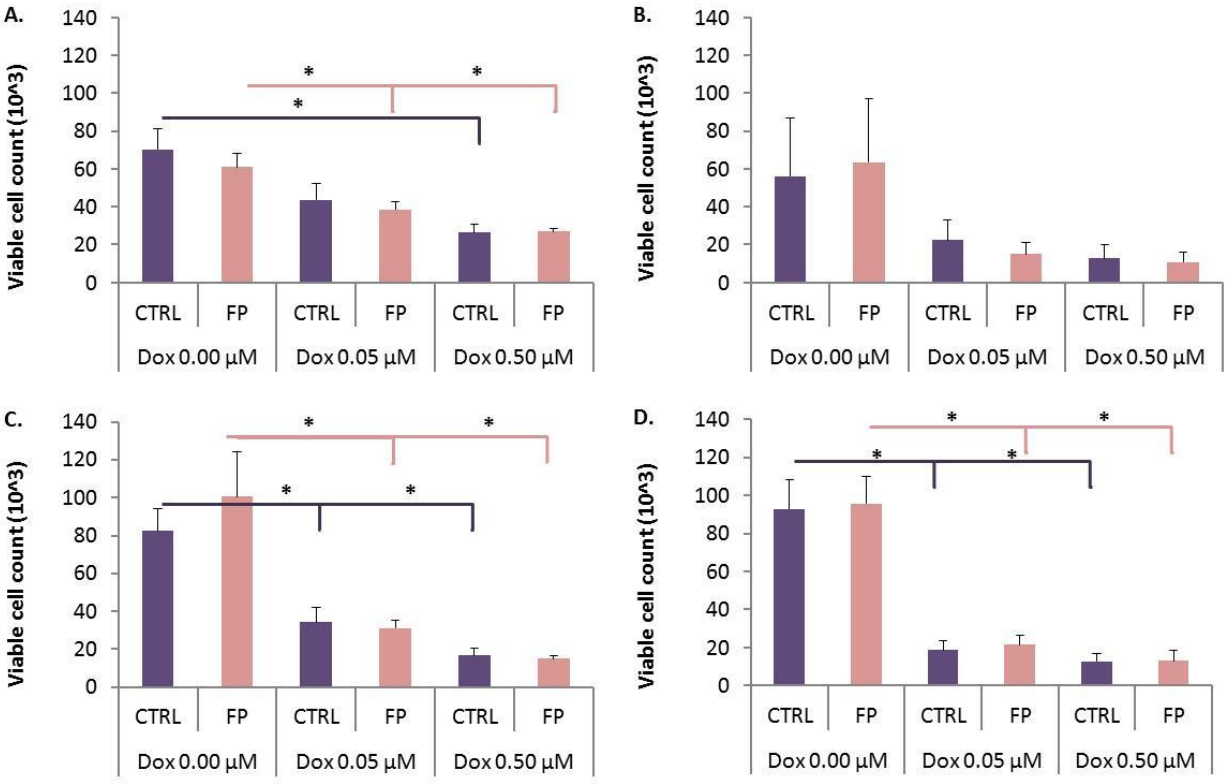


Figure 20: Cytotoxic response to Doxil of MDA-MB-231 cells passaged for 3 weeks in culture post surviving dual rounds of HIFU exposure at the focal point (FP) in comparison to mock controls (CTRL HIFU). The 2 rounds of HIFU application were performed 3 weeks apart; i.e. cells surviving round 1 were passaged for 3 weeks in tissue culture then

subjected to a second round of HIFU. Trypan blue vital stain exclusion assay was used to count viable cells starting with 24hr post Doxil treatment (0.05μM or 0.5μM) for 4 consecutive days (panel A: day 1; panel B: day 2; panel C: day 3; and panel D: day 4). Data represent mean ± SEM of three independent repeats performed in duplicates. Asterisks represent statistical significance ($p<0.05$).

CHAPTER IV

DISCUSSION

Cancer ablation by HIFU application hasgained growingattention by the medical community in recent years primarily due to its non-invasive procedure that potentially has fewer side effectsand favourable outcomes in comparison or in combination with standard therapeutic approaches. However, the effects of sub-lethal (sub-cavitation) HIFU exposure on cellular mechanotransduction and cytotoxic response toanti-neoplastic agents have not been examined.

In a previous study in our laboratory that was pursued in collaboration with the laboratory of Dr. Ghanem Oweis (Department of Mechanical Engineering, FEA, AUB), we designed a HIFU setup that mimics models of HIFU transducers used in previous reports (Yunbo Liu *et al*. 2005).The HIFU transducer (frequency of 2.158 MHz) was kindly provided by Dr. Mike Bailey's laboratory, CIMU, University of Seattle, USA. The HIFU setup was co-

designed and manufactured by Dr.GhanemOweis Laboratory, at the Department of Mechanical Engineering, FEA, AUB, in Beirut, Lebanon. Following parameter optimization, we examined the alterations in mechanotransduction due to changes in the physical properties resulting from the exposure of MDA-MB-231 breast cancer cells and MCF-10A immortalized mammary epithelial cells to ultrasonic waves from this custom-designed HIFU setup.In this follow-up study, we continued assessment of putative changes in the expression of four mechanosensitive genes and looked at the resultant consequences on cellular response to anti-neoplastic agents.

Overall, we assessed the *in vitro effects* of sub-lethal HIFU exposure on the expression of seven mechanosensitive genes namely *CAV-1* (Caveolin-1 α & β), *Hic-5* (Hydrogen Peroxide-Inducible Clone 5), *PXN* (Paxillin), *TTLL4* (Tubulin-Tyrosine Ligase-Like Protein 4), *TWIST1* (Twist-Related Protein 1), *CTSD* (Cathepsin D), and *HSPA1A* (Heat Shock Protein 70) whereby we quantified using Real-Time PCR significant enhanced expression of *CAV-1α*, *PXN*, and *Hic-5* that was immediate-early (1hr post HIFU exposure) in MCF-10A cells and delayed (24hr post HIFU exposure) in MDA-MB-231 cells. Additionally, we noted a significant immediate –early transient increase in *TTLL4* expression in both cell lines and in *TWIST1* expression in MDA-MB-231 cells. Notably, sub-lethal HIFU exposure had no significant effect on the expression of *CAV-1*(total pool; i.e. combined pool of α and β isoforms), *CTSD*, and *HSPA1A* in both cell lines at both time points (1hr and 24hr following sub-cavitation HIFU exposures). These results suggest that residual HIFU exposure results in reduced expression of the β isoform of caveolin-1 by favouring the expression of the *α* isoform since no significant change in the total caveolin-1

transcript pool was obtained.A plausible explanation for this is that the cells reduce splicing and generate less of the mRNA that encodes for the Cav-1β, thus making more of the α isoform. This could be attributed to stress (resultant from sub-cavitation) HIFU exposurewhich induces a shift in isoform formation that favours Cav-1α. A previous study reported that theα isoform of caveolin-1 responds to oxidative stress to ensure normal cell function (Sun *et al*. 2009).Interestingly, the β isoform of caveolin-1 has been associated with resistance to Taxol whereas the α isoform is believed to enhance cellular sensitivity to the cytotoxic effects of Taxol by promoting apoptosis.

As illustrated in the results section, there was no significant change in*TWIS1*gene expression in MCF-10A cells that were exposed to sub-lethal HIFU levels in comparison to mock-treated control at any of the two tested time points. However, MDA-MB-231 breast cancer cells placed ata distance of 42.5mm from the transducer (estimated to have received25.7% of the intensified energy at the focal point) showed a 4.5 (±0.62) – fold induction in *TWIST1*gene expression in comparison to mock control when tested at 1hr following HIFU exposure. No significant change in *TWIST1* expression was obtained in these cells at the 24hr time point suggesting that the enhanced expression was transient. Similarly, MDA-MB-231 breast cancer cells placed at a distance of 43.5mmfrom the transducer (estimated to have received75.7% of the intensified energy at the focal point) showed a 4.75 (±3.16) – fold induction in *TWIST1*gene expression in comparison to mock control when tested at 1hr following HIFU exposure. However, this result was not statistically significant likely due to the very high standard deviation and SEM, thus moreindependent repeats should be carried out to minimize experimental error. No

significant change in *TWIST1* expression was obtained in MDA-MB-231 cells subjected to either HIFU intensity at the 24hr time point suggesting that the enhanced expression measured at the 1hr time point was transient. Moreover, no significant change in *TWIST1* expression was obtained in MCF-10A cells subjected to either HIFU intensity at either time point tested.

Since *TWIST1* is a mechanosensitive gene implicated in breast cancer progression and in EMT, the observed enhanced expression of *TWIST1* in the mesenchymal-like breast cancer cells MDA-MB-231 post exposure to sub-lethal HIFU suggests that these cells may be triggered to undergo furtherEMT (Onder*et al*. 2008). However, more research should be done to decipher the effects of this immediate-early enhanced gene expression on EMT in MDA-MB-231 cells treated with HIFU and to determine if it were enough to enhance the metastatic potential of these cells by influencing the expression and function of other genes (Weiss *et al.* 2012). Accordingly, we propose to look at putative changes in other genes that are implicated in EMT and to examine MDA-MB-231 cells treated with HIFU in a 3D context that is more relevant to study enhanced invasive and metastatic potential. Additionally, we are interested in validating if sub-lethal HIFU exposure alters cells in 3-D matrigel cultures resulting in altered lumen formation.

CathepsinD was not significantly altered in both cell lines post HIFU exposure and at both time points compared to the control. This could be explained by the fact that *CTSD*gene expression is not affected by acoustic waves under the experimental conditions that were tested. Moreover, it is possible that more time points should be checked.

Likewise, Heat Sock Protein 70 expression was also not changed post HIFU exposure in both cell lines at both time points. In light of its implication in conferring resistance to Taxol and Doxil when enhanced in expression, it is of importance that *HSP70* was not elevated in the tested cell lines post sub-lethal HIFU exposure. Considering that *HSP70* is a chaperon protein responsive to heat, our data indicates that acoustic energy from the HIFU transducer which cells are exposed to under the experimental parameters applied in this study is of mechanical nature and not thermal.

Furthermore, another aim of this study was to test the *in vitro* effects of sub-lethal HIFU exposure on the cytotoxic response of MCF-10A and MDA-MB-231 cells to anti-neoplastic agents Doxil (Doxorubicin) and Taxol (Paclitaxel). These two chemotherapeutic drugs have variable modes of action which result in cellular cytotoxicity when applied at optimal concentrations in that Taxoldisrupts the microtubule network whereas Doxil acts on DNA. For the cytotoxicity assays, we selected two points for the treatment of HIFU exposed cells with either anti-neoplastic agent;the 6hr time point post HIFU exposure takes into account the timespan needed for putative changes in protein expression of mehcanosensitive genes that may have had immediate-early modulation in gene expression (i.e. 1hr post HIFU) whereas the 30hr time point post HIFUtakes into account the timespan needed for putative changes in protein expression of mehcanosensitive genes that may have had delayed modulation in gene expression (i.e. 24hr post HIFU). Accordingly, this would enable us to interpret the data that would be obtained under this aim in the context of results obtained under aim 1.

Our results indicate that both MDA-MB-231 cells and MCF-10Acells are sensitized to suboptimal cytotoxic doses of Taxol (1.5nM, 7.5nM) when the drug was added to cells 6hr or 30hr following sub-lethal HIFU exposure; i.e. post residual HIFU exposure of cells that are situated at a distance from the focal spot which receive an estimated 26% or 83% of the intensity at the focal point. Similarly, both MDA-MB-231 cells and MCF-10A cells are sensitized to suboptimal cytotoxic doses of Doxil (0.05μM, 0.5μM) when the drug was added to cells 6hr or 30hr following sub-lethal HIFU exposure. The rationale for using suboptimal cytotoxic concentrations of either drug is to enable us to determine if sub-lethal HIFU treatment would modulate cellular response to these agents by acting in synergy with chemo (sensitizing cells to Taxol or Doxil) or by being antagonistic (de-sensitizing cells to Taxol or Doxil).

In all performed experiments, we noted significant synergistic effects between HIFU exposures (at sub-cavitation levels) and suboptimal cytotoxic doses of Taxol or Doxil which could be attributed in part to the altered profile in mechanosensitive gene expression such as the enhanced expression of Cav-1α at the expense of reduced expression of Cav-1β. Another plausible factor that may be implicated in the observed enhanced sensitivity to Taxol and Doxil post sub-cavitation HIFU exposure is enhanced cellular uptake of either drug via multiple mechanisms including sonoporation.

Sonoporation refers to the formation of transient and/or irreversible pores on the surface of the plasma membrane induced by ultrasound exposure (Deng *et al.* 2004). These pores on the surface of the plasma membrane result in membrane permeabilization, hence allowing molecules, otherwise impermeable, to get inside the cell. Several studies have reported a

relation between ultrasound and enhanced drug uptake in that low intensity ultrasound was shown to induce drug endocyotosis whereas high intensity ultrasound was found to induce pore formation, hence direct drug uptake (Lentacker*et al*. 2014). In addition, sonoporation has been reported to impact the structure of organelles (Zeghimi*et al.* 2012). Hence, it is plausible that sub-lethal HIFU exposure alters the structure and/or number of membrane caveolae in cells.

Moreover, we rationalize that alterations in the phosphorylated forms of proteins expressed by select mechanosensitive genes may contribute to the enhanced sensitivity of mammary epithelial cells and breast cancer cells to chemo post sub-lethal HIFU exposure.

Given that sub-lethal HIFU exposure sensitizes MDA-MB-231 and MCF-10A cells to sub-cytotoxic doses of Taxol and Doxorubicin, we are next interested in deciphering the mechanisms that are implicated in this enhanced *in vitro* sensitivity of either cell line to anti-neoplastic agents in order to provide a mechanistic insight into the results obtained in our laboratory. Additionally, we are interested in validating if this enhanced sensitivity to chemo also applies to 3-D cultures.

Furthermore, our results show that MDA-MB-231 cells surviving single or dual rounds of HIFU exposure at the focal point and passaged over 3-to-6 weeks in tissue culture showed no significant change in their *in vitro* sensitivity to Taxol or Doxil, suggesting that the cells did not develop resistance when passaged over time. The rationale for passaging the cells which survived HIFU exposure at the focal point for successive weeks in tissue culture is to permit for selection of cells that may potentially have developed resistance to either one or

both chemotherapy agents. It is plausible that cells surviving HIFU at the focal point may be more tolerant to the tested doses of Doxil and/or Taxol. Additionally, surviving cells may have acquired *de novo* mutations as a result of cellular deformations that may have resulted from exposure to HIFU at the focal point.

In conclusion, sub-lethal HIFU exposure *in vitro* resulted in significant alterations (immediate – early or delayed) in the expression of select mechanossensitive genes in MCF-10A and MDA-MB-231 cells and sensitized both cell lines to suboptimal cytotoxic doses of Taxoland Doxil when added 6hr or 30hr post HIFU treatment. Moreover, MDA-MB-231 cells surviving single or dual rounds of HIFU exposure at the focal point showed no significant change in their *in vitro* sensitivity to suboptimal cytotoxic doses of TaxolandDoxil indicating that the cells did not acquire drug resistance. Next, we propose to determine if sonoporation is implicated in the enhanced *in vitro* sensitivity of MCF-10A and MDA-MB-231 cells to suboptimal cytotoxic doses of Taxol andDoxil post sub-lethal HIFU exposure. Additionally, we aim to assess post-translational changes in phosphorylation of Cav-1 (α isoform), in Hic-5, Paxillin, and TTLL4 in both cell lines post *in vitro* exposure to sub-lethal HIFU and determine if both cell lines exhibit changes in lumen formation and/or cellular polarity in 3-D matrigel post *in vitro* exposure to sub-lethal HIFU. Moreover, we intend to examine if MDA-MB-231 cells surviving single or multiple rounds of lethal HIFU exposure *in vitro* display elevated levels of cancer stem cell markers.

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