Combination Chemo and Hyperoxia Therapy in Breast Cancer Cells using Nanoemulsion Delivery Systems

Masters of Science Thesis

By

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SUMMARY

Despite significant advances in new drug discoveries and treatment combinations, the mortality rate due to cancer has not changed significantly over the last fifty years in the United States and many parts of the world. The inability to detect cancer in its early stage accounts for the lethality, which is then associated with poor prognosis as a result of dissemination to other organs. In breast cancer, for instance, current therapeutic strategies fail to remove root cause of the diseased. This is largely because of the insufficient accumulation of chemotherapeutic agents into the tumor site upon systemic administration and micro environmental selection pressures leading to development of multidrug resistance. As such, there is a critical need to develop more effective therapeutic strategies for breast cancer that lead to enhanced clinical benefits of anticancer therapeutics without the associated systemic toxicity.

Upon initiation of angiogenesis and recruitment of neovasculature, there are regions of solid tumor that lack blood supply. These regions are characterized by the hypoxia, which has been implicated to provide a selective niche for alteration of tumor cell phenotype leading to resistance. Hypoxia induces the expression of hypoxia inducible factor (HIF), which acts as the transcriptional activator of other genes important in disease aggressiveness and metastasis. These include genes that regulate cell proliferation, angiogenesis, glucose transport and metabolism, and cellular invasion from primary tumor.

Based on the need to develop strategies that can alter tumor hypoxia-induced changes, the objective of this study is to evaluate the reversal through hyperoxia treatment in combination with a drug delivery system that can increase tumor drug concentrations, residence, and intracellular delivery. This study evaluated the role of oil-in-water nanoemulsion formulation
encapsulating a hydrophobic derivative of doxorubicin, an anthracycline anticancer agent, on cytotoxicity and apoptotic enhancement of tumor cells treated under different oxygen conditions.

Nanoemulsion formulations using fish oil, which is rich in omega-3 polyunsaturated fatty acids, as the internal hydrophobic phase, show rapid intracellular accumulation of doxorubicin stearate (DOX stearate) in the endosomes which acts as a reservoir for the release of free DOX over period of time. Increased levels of the reactive oxygen species were detected in the cells treated with 40 and 60% hyperoxia which aid in the cell killing efficiency of DOX stearate. Also, the cytotoxicity and apoptotic activity of doxorubicin stearate was evaluated when administered in nanoemulsion formulations to cells incubated under different oxygen conditions. The data showed enhanced cell killing efficiency of the drug at lower drug concentration. Using MDA-MB-231 triple negative human breast adenocarcinoma cells, we evaluated the changes in expression profile of gene and protein levels of HIF-1α upon treatment with hypoxia, normoxia, and hyperoxia conditions. Western blot analysis did show degradation of HIF-1α at increase oxygen level but needs to be proved in a better way. Lastly, the results of this study provided important guidance on the clinical utility of hyperoxia therapy in breast cancer patients.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>2</td>
</tr>
<tr>
<td>ACKNOWLEGEMETSS</td>
<td>6</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>7</td>
</tr>
<tr>
<td>List of Tables</td>
<td>8</td>
</tr>
<tr>
<td>List of Figures</td>
<td>9</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>11</td>
</tr>
<tr>
<td>2. OBJECTIVES AND SPECIFIC AIMS</td>
<td>35</td>
</tr>
<tr>
<td>3. EXPERIMENTAL DESIGN AND METHODS</td>
<td>39</td>
</tr>
<tr>
<td>4. RESULTS AND DISCUSSION</td>
<td>52</td>
</tr>
<tr>
<td>5. CONCLUSIONS</td>
<td>69</td>
</tr>
<tr>
<td>6. REFERENCES</td>
<td>70</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

**Table 1:** Particle size and surface charge characterization of the blank and doxorubicin stearate containing oil-in-water nanoemulsion formulations.

**Table 2:** Encapsulation efficiency of doxorubicin stearate in oil-in-water nanoemulsion formulations.
LIST OF FIGURES

Figure 1: Estimated new cases and deaths due to different types of cancer in male and female in the United States. Marked circle shows the percentage of estimated new cases and death in female due to breast cancer.

Figure 2: Annual rates of diagnosis of each type of cancer in male and female in United States. Marked circle shows the diagnostic rate of breast cancer in female.

Figure 3: Annual rates of deaths in female due to different types of cancer.

Figure 4: Process of physiological angiogenesis.

Figure 5: Types of cellular mechanisms involved in tumor angiogenesis.

Figure 6: Process of oncogenes-induced tumor angiogenesis.

Figure 7: Process of angiogenesis in avascular tumor.

Figure 8: Newly formed tumor blood vessel having tortuous shape, thin walled without pericytes, where green cells are normal tumor cells and black cells are necrotic tumor cells.

Figure 9: Oxygen-dependent production and degradation of HIF.

Figure 10: Mechanism of increased concentration of oxygen, in hyperoxia, on HIF-1α.

Figure 11: Mechanism of passive tumor targeting.

Figure 12: Mechanism of active tumor targeting through receptor-mediated endocytosis.

Figure 13: $^1$H-NMR of the Doxorubicin Hydrochloride solution (A), Sodium Stearate (B) and Doxorubicin Stearate Nanoemulsion (C).

Figure 14: Transmission electron microscopy images of the (A) Blank and (B) Doxorubicin stearate encapsulated nanoemulsion formulations negatively stained with uranyl acetate.

Figure 15: Confocal microscopy images showing the cellular uptake of Doxorubicin Hydrochloride, Doxorubicin Stearate solution and Doxorubicin Stearate nanoemulsion in MDA MB 231 cells followed by 6 hrs of incubation. Panel’s A-E-I, B-F-J, C-G-K and D-H-L shows uptake under hypoxic, normoxic, 40% and 60% hyperoxic conditions respectively. Magnification used was 40X.
**Figure 16:** Intracellular Drug uptake studies carried out in combination of DOX stearate and oxygen therapy. A-D shows the levels of the drug uptake in MDA MB 231 cells under hypoxia, normoxia, 40% and 60% hyperoxia.

**Figure 17:** Reactive oxygen species detection assay to determine the levels of highly reactive oxygen species under different oxygen incubation conditions.

**Figure 18:** Cytotoxicity studies carried out in combination of DOX stearate and oxygen therapy. A-D shows the cell viability graph and IC50 values of the drugs under hypoxia, normoxia, 40% and 60% hyperoxia.

**Figure 19:** Caspase-3/7 activation, relative to untreated cells, in MDAMB 231 cells following treatment with doxorubicin solution (DOX), doxorubicin stearate solution (DOX St), doxorubicin stearate in nanoemulsion in hypoxia, normoxia, 40% and 60% hyperoxia (A-D). The results represent mean (n = 3) along with standard deviation.

**Figure 20:** TUNEL staining images of untreated MDA MB 231 cells in the normoxic conditions.

**Figure 21:** TUNEL staining images of treated MDA MB 231 cells in the hypoxic conditions.

**Figure 22:** TUNEL staining images of treated MDA MB 231 cells in the Normoxic conditions.

**Figure 23:** TUNEL staining images of treated MDA MB 231 cells in the 40% Hyperoxic conditions.

**Figure 24:** TUNEL staining images of treated MDA MB 231 cells in the 60% Hyperoxic conditions.

**Figure 25:** Gel electrophoresis of the RT-PCR product of HIF-1α to determine its expression at mRNA level under different oxygen conditions.

**Figure 26:** Determination of relative expression of the HIF-1α at mRNA level under different oxygen conditions using HIF-1α specific qPCR array.

**Figure 27:** Down-regulation of HIF-1α by western blot analysis.
1. INTRODUCTION

1.1. Breast Cancer

Cancer is one of the major health diseases in United States and in other parts of the world. In United States, total deaths due cancer is 569,490 with 299,200 deaths in male and 270,290 in female occurred in 2010. Also, estimated new cancer cases in female are 739,940 and 789,620 in male occurred in year 2010 [1]. 1 out of 4 people in United States die because of cancer. Though death rate, due to cancer, of male and female decreased by 18.4% and 10.5% in 2004 as compared to that in 1991, the disease still accounts for more death in people of under the age of 85 years than heart disease [2].

Breast cancer is a leading cause of morbidity and mortality, especially in young pre-menopausal women of age 30-50 years. It is the malignant form of cancer originating from breast tissues especially from ducts and lobules. Cancer originating from ducts and lobules are called ductal carcinoma and lobular carcinoma, respectively. Breast cancer cause due to the up-regulation of hormone estrogen are called estrogen receptor.

Figure 1: Estimated new cases and deaths due to different types of cancer in male and female in the United States. Marked circle shows the percentage of estimated new cases and death in female due to breast cancer.
positive or ER positive cancer and are less aggressive cancer tumors. Breast cancers that involves genetic changes like down regulation of tumor gene suppressor genes 1 (BRCA 1) and breast cancer gene 2 (BRCA 2) or up regulation of HER2, regulates cellular growth and division, are more aggressive [3].

A large number of women are affected than male population from the breast cancer. In 2008, from the total 184,450 new breast cancer cases, 182,460 were found in female and out of 40,930 deaths from breast cancer 40,480 were in female [2]. In terms of estimated new cases breast cancer accounts for 26% of total cases in female for all leading cancer types in 2008. Also, breast cancer accounts for 15% of the total death in female from all leading cancer types [2] as shown in Figure 1. The incidence rates of the breast cancer in female as compared to male are highest among all other cancer types as shown in Figure 2. Also the annual death rates because of breast cancer are second highest in female among all other cancer types as shown in Figure 3.

Figure 2: Annual rates of diagnosis of each type of cancer in male and female individuals in the United States. Marked circle shows the diagnostic rate of breast cancer in female.
1.1.1a. Types of Breast Cancer: Following are some of the main types of Breast cancer [4]:

1. **Invasive Ductal Carcinoma (IDC):** IDC is the most frequently occurring breast cancer. It originates in from ducts and gradually infiltrates into surrounding tissues. Tumor cells vary in shape such as sheets, nodules or glandular and size from small to large. About three fourth of the total carcinomas found are Invasive Duct Carcinoma. It accounts for 55%-65% 5 year survival rate [4].

2. **Mucinous Carcinoma:** This is a type of IDC in which well differentiated small groups of tumor cells afloat in extra luminal, extracellular mucus. This mucus is in direct contact with the surrounding connective tissues. Because of its appearance, it is also known as Colloidal Carcinoma. As they are well differentiated prognosis can be easily done

3. **Medullary Carcinoma:** This is a less common form of IDC. Here the tumor cells are larger and spherical in shape, different from healthy cells in tissue. The color of the tumor mass is that of medulla in brain. This particular carcinoma is easy to diagnose.
4. **Tubular Carcinoma:** This is also a rare type of IDC. It is marked by the presence of tubules in tumor mass. These tubules are single layer of small tumor cells. These types of tumor are considered premature and are supposed to acquire aggressive nature in later stages.

5. **Inflammatory Breast Cancer:** It is one of the aggressive carcinoma that leads to inflammatory response by blocking lymph vessels of the skin in breast. It causes reddening and swelling of the breast. It is one of the rapidly growing forms of breast carcinoma.

1.1.1b. **Stages of Breast Cancer:** Breast cancer is divided into five stages for diagnosis and therapy as follows:

**Stage 0:** In this stage, cancer cells are localized in either ducts or lobules and not into the surrounding tissues, also referred to carcinoma *insitu* stage. This stage is highly treatable and five year survival rate is 100% [5]. It can be either Ductal or lobular carcinoma *insitu*.

**Stage 1:** Here the cancer cells are localized to their respective sites and size of the tumor is not more than 2 centimeters.

**Stage 2:** This stage is divided into two types depending upon the size of tumor and whether it had spread to the surrounding tissues or not. In Stage 2A and 2B size of tumor is between 2-5 centimeters and greater than 5 centimeters respectively and might be spread till three auxiliary lymph nodes [5].

**Stage 3:** This stage is also divided into two types. In stage 3A tumor spreads till 9 axillary lymph nodes with size ranging from 2-5 centimeters. In stage 3B tumor metastasize to the nearby tissues like chest, muscles, ribs [5].

**Stage 4:** In this stage metastasis occurs in major organs of the body such as lung, bones and liver. The five year survival rate is only 16% in this stage [5].
1.1.1c. Breast Cancer Metastasis and Prognostic Markers: Breast cancer is a highly aggressive form of cancer that initially starts with localized primary tumor and slowly metastasizes into the other distant organs. 10-15% of the patient have an aggressive form of breast cancer that metastasizes into other organs 3 years after detection of primary tumor [6]. Lungs, bones and liver are the main sites of the metastasis. Various theories have been proposed to known the etiology of breast cancer metastasis whether it is inherent property or acquired late during the tumor development due to genetic manipulations. One such model explains that oncogenic mutations in the breast cancer cell results into either “non metastatic, good diagnostic” stem cell that leads give rise to localized tumor or into “highly metastatic, poor diagnostic” stem cells which give rise to systemic breast cancer [6]. Different prognostic markers are established to determine the metastatic capacity of the primary tumor. Traditional markers such as tumor size, axillary lymph node status and histological grade are followed by the clinicians to diagnose the metastatic capacity of tumor in early stages. Tumor size is important independent prognostic marker, breast tumor mass with 2-5 centimeters or above 5 centimeters is considered to be associated with high risk of metastasis. Axillary lymph node and histological grade markers are dependent on tumor size. Presence of 4 or more lymph node metastases are consider to be acquire high risk of metastasis and are described by histological grade 3 tumors. However poor prediction of metastatic risk of the tumor mass by this traditional markers results into either inadequate or cytotoxic chemotherapy in patient with breast cancer. So, certain new prognostic markers have been developed such as gene expression profiling that can successfully scan the genome of the tumor mass and can predict metastatic property, site of metastasis and type of chemotherapy. Over expression of epidermal growth receptor factor-2 (ERBB2) or
human epidermal growth factor receptor-2 (HER2/neu) is associated with high risk of metastasis in lymph node positive tumors [6].

1.1.1d. Current Treatment Options: Various modes of treatment are surgery, radiation hormone and chemotherapy. However, instead of individual therapy, a combination therapy is widely used as it is more effective.

(a) Surgery: This is the best suitable option for patient with primary breast cancer. After surgery the remaining tumor can be treated by either radiation or chemotherapy. The different types of surgeries involved can be classified as follow:

- Lumpectomy: It involves the removal of only tumor mass of the breast. Nowadays it is more preferable surgery option for patients.
- Mastectomy: It involves the removal of the entire tissue of the breast along with the removal of tumor mass.
- Axillary lymph node dissection: The entire axillary lymph node is removed if the patient has metastasized tumor in lymph node. However, it caused loss of lymph nodes in the patient with negative axillary lymph node tumors and can result into death. So, Sentinel-lymph-node mapping is developed in which, only part of the lymph node is removed and checked for metastasis [3].

(b) Radiation Therapy: Ionizing radiations are used either to destroy or impede the tumor growth. The risk of recurrence of the tumor decreases by 50 -75% on administration of radiotherapy after surgery. There are two types of radiation therapy.

- External radiation therapy: External source of radiations is used to impart radiations on the affected area of the body.
Internal radiation therapy: A radioactive substance sealed in needles, seeds, wires, or catheters is either placed directly into the tumor area or is placed nearby [3].

(c) Hormone Therapy: This therapy is used for the patient with ER positive tumors. Combination of hormone and chemo therapy reduces the risk of recurrence and death by more than 15 years. Tamoxifen is used as an inhibitor of estrogen receptor and block the activity of over produced estrogen. It is administered for more than five years. Tamoxifen therapy is used as an adjuvant therapy with other chemotherapy [3].

(d) Chemotherapy: Several chemotherapeutic agents are used either in combination with hormonal therapy or in multi drug therapy. Multi-drug (“cocktails”) therapy has been shown to decrease the projected annual deaths by 20% as compared to single drug therapy. Combinations of the drugs that are most often used are fluorouracil, doxorubicin, and cyclophosphamide (FAC); 5-fluorouracil, epirubicin, and cyclophosphamide (FEC); doxorubicin and cyclophosphamide (AC); cyclophosphamide, methotrexate, and 5-fluorouracil (CMF). Standard therapy includes 6 cycles of FAC or FEC, 6 cycles of CMF or 4 cycles of AC administered at interval of 3-4 weeks.

For metastatic breast cancer once the diagnosis has been done, therapeutic strategy is done based on the location and extent of metastases; also it is depends on the age, hormone-receptor status and disease free interval state of women. CMF or FAC are used as first line of treatment. Taxanes such as paclitaxel (Taxol®) and docetaxel and Vinca alkaloids such as Vinorelbine (Navelbine®) has been as second and third line of treatment. Also, new therapeutics that is under development is liposomal doxorubicin (Doxil®), gemcitabine (Gemzar®) and anti-folic agents, like methotrexate [3].
1.2. Tumor Blood Supply and Hypoxia

1.2.1. Vascular Supply in the Body: The process of transporting oxygen and nutrients occurs via vascular system. The growth of vascular system in the body is regulated by two processes vasculogenesis: formation of new blood vessels and angiogenesis: highly regulated physiological process resulting in formation of new capillaries from existing vessels. Vasculogenesis on the other hand can function unregulated in cancer and inflammatory disease [7].

The body requires oxygen and nutrients to survive on daily basis. Blood fulfills this requirement by transporting them to different organs of the body via blood vessels and capillaries. Process of angiogenesis is initiated when oxygen availability is low i.e. hypoxia in certain region of the body. To counteract hypoxia cell stimulates hypoxia inducible factors (HIFs), guanylate cyclases, endoplasmic reticulum associated kinases and mTOR [7]. These factors maintain the oxygen balance via formation of new blood vessels. Angiogenesis involves restructurization of capillaries, micro vascular growth and fusion into mature vessels in vascular and avascular region of the tissues. It involves complex interaction with endothelial cells, extracellular matrix, stromal and pericytes cells and finally with basal membrane. Angiogenesis starts with the opening of the capillaries thereby allowing cell infiltration. HIF 1α expression in the endothelial cells increases vascular endothelial growth factor A (VEGFA) and vascular endothelial growth receptor factor 2 (VEGFR2). VEGFR2 mediates transportation of endothelial cells in VEGFA macromolecules into the avascular region. Potentiating of the VEGFR2 activity occurs because of the interaction of VEGF and neurophilin- 1 (NRP1). Other forms of VEGF A such as VEGF B, Placenta growth factor (PLGF) interacts with VEGFR1, whereas VEGF C and VEGF D initiated lymph angiogenesis via interaction with VEGFR3. Increased VEGF A
concentration leads to the formation of highly motile tip of endothelial cells that proliferates to give rise to new vessel sprouts. Tip endothelial cells acquire proteolytic phenotype that degrades basal membrane resulting into exposure of cells to extracellular matrix. In the meantime, membrane type -1 matrix metalloproteinase (MT1-MMP) expressed on endothelial cells breaks apart extracellular matrix to guide the movement of endothelial cells. They also help in formation of lumen and vacuoles in endothelial cells. VEGF stimulates the expression of delta like ligand 4 (DLL 4) on endothelial cells. This ligand binds to the notch receptors 1 and 2 of the neighboring tips of the endothelial cells. This signals the down regulation in further movement of endothelial cells and promotes vessel development. This resolution phase initiates the production of pericytes and vascular smooth muscle cells (VSMCs) mediated by the secretion of platelet-derived growth factor-BB (PDGF-BB) from the endothelial cells. These cells express angiopoietin 1(ANGPT1) that binds with the TIE2 receptors expressed on the endothelial cells. This binding results into the remodeling and blood vessel stabilization. The stabilization of vessels results into the induction of tissue inhibitor of metalloproteinases 2 (TIMP 2) in endothelial cells and tissue inhibitor of metalloproteinases 3 (TIMP 3) in pericytes, which inhibits proteolytic activity of endothelial cells. Finally, complete vessel formation leads to blood flow in that particular region increasing oxygen concentration and thereby decreasing VEGF levels in endothelial cells.

**Figure 4:** Process of physiological angiogenesis.
This marks the completion of angiogenesis [7]. **Figure 4** describes the process of angiogenesis.

**1.2.1b. Tumor Angiogenesis:** The main difference between tumor and normal angiogenesis is that in case of cancer the angiogenic process in continuous and highly unresolved due to tumor hypoxia and its different secreted factors [7]. The normal process of angiogenesis occurs in due balance of pro- and anti angiogenetic factors. However, in order to grow further and metastasize, tumor cells shifts the above balance towards pro angiogenetic factors. This transition phase of tumor cells towards angiogenesis is known as *angiogenic switch* [8]. Various factors that accounts for this switch are proliferating cells, low pO$_2$, inflammatory response, genetic manipulations like increased expression of oncogenes and decreased expression of tumor suppression genes. All these factors are secreted from different cells like endothelial cell, cancer cells, connective tissue cells and from extracellular matrix [9]. Formation of new tumor vessels occurs via different ways such as sprouting, intussusceptions i.e. formation of interstitial columns by existing tumor vessels into the lumen of existing vessels or by incorporation of endothelial precursors as shown in **Figure 5** [9].

Process of sprouting is the same as it occurs in normal physiological angiogenesis. First an avascular tumor grows till inner core becomes hypoxic and induces expression of hypoxia inducible factors in tumor cell. These lead to the
increased expression of VEGF and other angio genetic factor such as fibroblast growth factor (FGF), Interleukin-8 (IL-8). VEGF and FGF initiates angiogenic cascade by vessel destabilization. An activated fibroblasts i.e. stromal cell secretes other vascular growth factors like MMP, VEGFA, VEGFC, FGF2 and inflammatory factors such as Granulocyte colony stimulating factor (GCSF) and Platelet derived growth factor C (PDGFC). These lead to failure in angiogenic resolution resulting into continuation of angiogenic process as shown in Figure 6 [7, 10]. This factors are also responsible for the migration of the endothelial precursors into the tumor vessels [9]. However if tumor cells grows on the pre-existing vessels they secrete Angiopoietin 2 (ANG2) which causes endothelial cell apoptosis. This apoptosis results into suppression of the existing vessel leading to hypoxic environment as shown in Figure 7. So, tumor is now known as avascular tumor and it initiates angiogenesis as described above [10]. Tumor vessels thus formed are thin walled, highly mosaic, leaky structures with many gaps and absence of pericytes as shown in Figure 8. As tumor growth and its metastasis are solely dependent on angiogenesis, it plays a critical role in tumor targeting [11].

1.2.2. Hypoxia: Hypoxia is the pathological condition in which oxygen levels in the body or certain regions in the body are very low. It plays an important role in maintaining homeostatic
mechanism of body by regulation of angiogenesis. These homeostatic mechanisms are the link between vascular oxygen and various other metabolic processes [12]. Hypoxia induces the expression of hypoxia inducible factor-1 (HIF-1) in normal and tumor cells. HIF-1 is the transcription activator of various genes involved in cancer biology such as angiogenesis (VEGF), glucose metabolism (GLUT-1, GAPDH, hexokinase 1 and 2), cell survival (insulin like growth factor 2, nitric oxide synthase 2) and invasion (cyclin G2, transforming growth factor α and β3).

Also HIF-1 upregulates the expression of genes involved in extracellular matrix metabolism such as MMP2, Urokinase plasminogen activator receptor (UPAC) and cathepsin D (CATHD) [13]. HIF-1 expression mediates the adaptive changes in response to the reduced tissue oxygenation. HIF-1 is the heterodimer transcription activator protein having basic helix-loop-helix-PAS domain. It has two subunits one is HIF-1α subunit which is highly regulated and the other subunit is HIF-1β which is constitutively expressed nuclear proteins [14]. Synthesis of HIF-1α is oxygen independent and degradation is oxygen dependent.

Synthesis of HIF-1α occurs via phosphotidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways. Now mTOR and ERK produce via PI3K and MAPK pathways respectively phosphorylates p70-S6 kinase, which in turns phosphorylates ribosomal proteins and eukaryotic translation initiation factor- 4E (eIF 4E). This were increase in rate of
translation of HIF-1α mRNA into protein [13]. On the other hand, degradation of HIF-1α is oxygen regulated process. Under normoxic condition, oxygen dependent hydroxylation of proline residues 402 and 564 on HIF-1α protein occurs via prolyl hydroxylase domain (PHD) 1-3 enzyme. These lead binding of von Hippel-Lindau tumor suppressor protein (VHL) on the HIF-1α resulting into the ubiquitination of HIF-1α through the ubiquitin protein elongin C. Also, hydroxylation of asparagaine residue 803 on HIF-1α results into the binding of Factor Inhibiting Hypoxia-1 (FIH-1) which ultimately blocks the binding of HIF-1α with p300 and CBP. This inhibits the transcription mediated gene expression [14]. On the other hand, in hypoxic condition availability of molecular oxygen is less resulting into the decrease in hydroxylation rate of proline residues and asparagines. This inhibits binding of VHL to HIF-1α which leads to decrease HIF-1α degradation. Also, HIF-1α mediated gene expression is increased as binding of p300 and CBP occurs [13, 14].

**Figure 9** shows regulation of HIF-1α via oxygen. Genes targeted by HIF-1 depends upon various factors such as identification of hypoxia response element that has HIF-1 binding sites, inhibition of HIF-1 via interference RNA, over expression of HIF-1 in VHL null cells. However, factors associated with angiogenesis, cell survival and proliferation, glucose transporters and glycolytic enzymes are highly influenced by HIF-1 transcription activity [13].
Apart from physiological angiogenesis, *HIF-1* plays an important role in tumor growth and its angiogenesis. Up regulation of *HIF-1* system occurs via different growth stimulating factors like insulin growth factor-1, epidermal growth factor by two synthetic pathways discuss above. Also, oncogenic mutation in tumor suppressing genes such as p53 and PTEN and VHL encoding gene results in high up regulation of *HIF-1* system. Moreover, *HIF-1* system increases tumor growth via autocrine stimulation of various growth factors. Hypoxia induces expression of *HIF-1α* which in turn increases expression of growth factors like insulin like growth factor-2, Transforming growth factor-α [12, 13]. Nevertheless, hypoxia induced activity of *HIF-1α* depends on cancer type and the cells specific. Because, target genes activation is dependent on the interaction of *HIF-1α* and other transcriptional factors present in that particular hypoxic cells. Also, it has been shown that *HIF-1* system increases apoptosisis in some tumors and decreased mortality in other tumors which depends upon the genetic mutation caused by that particular cancer [13].

![Figure 9: Oxygen-dependent production and degradation of HIF.](image-url)
1.3. Role of Hyperoxia in Enhancing Cytotoxic Chemotherapy

Currently, systemic chemotherapy is the only viable approach used widely for the treatment of cancer. Usually combinations of various anti-cancer therapies are being used to overcome and treat resistant tumor cells. However, in majority of cases, these drugs are unsuccessful in the complete eradication of cancer. There are certain biological aspects of cancer and its growth that are to be understood. One such aspect is the hypoxia and its effect on tumor cells. To overcome this, research in area of hyperoxia has been initiated and is one of promising future therapy in way that it affects the core biology of cancer. Hyperoxia is the condition in which oxygen level or partial pressure of oxygen increases in tissues of the body. This idea is applied onto the hypoxic tumor cells to counteract the effect of hypoxia inducible factor-1. Measurement of the tumor oxygenation divided tumor cells into different layers depending on oxygen level, the center being necrotic and edge being hypoxic [15]. This gradient in oxygen in tumor cells has varied effect on various cellular processes. One way to overcome this is to supply more oxygen than ambient oxygen (21%) in to the mammalian tissues. This way, the molecular oxygen into tumor cells can be increased, which is required for different aerobic metabolism.

1.3.1a. HIFs as an Oxygen Sensor in Cancer: The impact of hypoxia inducible factor-1 (HIF-1α) is highly dependent on the level of molecular oxygen in the tumor cells. Studies carried out to understand the mechanism of HIFs during the changes in cellular oxygen revealed that HIF-1α subunit is highly regulated by an oxygen dependent degradation domain consisting of 200 amino acids [15]. Also, it was revealed that the hydroxylation and VHL dependent poly-ubiquitylation is reduced in hypoxic cells, resulting into the increase expression of HIF-1α and its target genes. Also, the interaction of HIF-1α with the factor inhibiting hypoxia-1 (FIH-1) reduces due to decreased hydroxylation of asparagines residue in hypoxia. However, HIF-1α’s
ubiquitylation and degradation is increased in oxygen replete cells i.e. hyperoxia via proteosome. This is because prolyl hydroxylase domain enzyme required for degradation and Factor inhibiting hypoxia-1 are dependent on oxygen, iron and 2-oxoglutarate [15]. In hyperoxia increased availability of molecular oxygen leads to tumor oxygenation thereby decreasing oxygen gradient across the tumor mass [16]. The proposed mechanism of reversal of the hypoxia induced $HIF-1\alpha$ activity is increased hydroxylation, poly-ubiquitylation and degradation of $HIF-1\alpha$. Increased availability of molecular oxygen causes the activation of prolyl hydroxylase domain (PHD domain) enzyme 1-3 and other hydroxylase. These lead to the hydroxylation of proline residues and asparagines residue resulting into the binding of Von Hippel-Lindau ($VHL$) and Factor Inhibiting Hypoxia-1 ($FIH-1$) respectively. Binding of $VHL$ and $FIH-1$ were lead to the poly- ubiquitylation and inhibit binding of $HIF-1\alpha$ with transcriptional co-factors p300 and CBP respectively [14, 15]. Figure 10 shows the effect of increased oxygen on inhibition of HIF activity.

Also, it has been proved that the activities of HIF-1 proteins are inversely related to the cellular oxygen concentration. The DNA binding activity and the expression of $HIF-1$
proteins: \textit{HIF-1}α and \textit{HIF-1}β increases exponentially with the decreasing oxygen concentration [17]. It was shown that the \textit{HIF-1}α and \textit{HIF-1}β RNA and proteins level decreases immediately on exposure of cells to increased oxygen concentration. \textit{HIF-1}α induced in turn binds to HIF-1 binding sites within the promoter region of the various genes that encodes \textit{VEGF}, \textit{GLUT-}, \textit{glycolytic enzyme and nitric oxide synthase}, which induces the hypoxia mediated transcription. However, the functional characterization of HIF-1 as an oxygen sensor in tumors is cell dependent [17].

1.3.1b. Effect of Hyperoxia on Tumor Cell Growth: There exists the oxygen gradient across the area of the tumor region resulting in lower HbO$_2$ saturation in this area. Also, Hypoxia inducible factor - 1α is main regulatory factor responsible for the various changes induced by hypoxia in tumor cells. This can be overcome by exposing the cells to higher amount of oxygen. These increase the partial pressure of oxygen in various tissues, enhancing the solubility of oxygen in the blood. The increase in the solubility of oxygen causes access of oxygen to previously obstructed physiologically areas leading to the increased Hb02 saturation. This were cause the retardation in the tumor growth via reduction in the expression of various glandular secretory proteins [18]. The expression of genes encoding different glandular proteins such as \textit{parotid secretory proteins (Psp)}, \textit{common salivary protein 1 (Csp1) and prolactin induced gene (Pip)} which are involved in the growth of the tumor, is down regulated on exposing tumor cells to hyperoxia. Also, hyperoxia marks the transition of mesenchymal cells to epithelial cells in tumor via inducing the expression of makers such as \textit{fibroblast growth factor receptor-1}, \textit{transforming growth factor α, β1 and β2}. Also, the retardation in tumor growth is being accompanied by the shifting of the glycolytic metabolism to oxidative phosphorylation via down regulation of enzymes such as \textit{hexokinase 2 and lactate dehydrogenase} [16]. Reduced
expression of these genes resulted into marked decrease in tumor size and tumor morphology [16, 18].

1.3.1c. Effect of Hyperoxia on Tumor Blood Flow: The decreased in the growth of tumor is accompanied by the anti-angiogenic effect of the increased amount of oxygen. There has been contradictory effect of hyperoxia on the normal and tumor cells. It induces the increase in the vessel development during wound healing in normal tissues. However, it causes reduction in the vascular density in tumor cells [16, 18]. The increased partial pressure of oxygen in the tumor tissue causes the degradation of HIF-1α and other vascular growth factors. Also, down regulation of genes such as VEGFα and VEGFβ, Fibroblast growth factor and platelet derived growth factor leads to reduction in the angiogenesis [16]. This resulted in the vasodilatation of the peripheral and central tumor vessel which increases blood flow into the tumor and prevents starvation by counteracting hypoxia [18].

1.3.1d. Mechanisms of Hyperoxia-Induced Cell Death: All the above effect caused by the hyperoxia is via proposed mechanism of apoptosis. The tumor suppressor gene p53 is main regulatory mechanism for induction of apoptosis, which after activation induces expression of other pro-apoptotic factors like PUMA, BAX, BAK, BIK AND PERP in tumor cells [16]. All these genes play cell specific role in wt-p53-induced apoptosis. Out of all these genes, Perp that encodes protein for epithelial integrity shows marked increases after hyperoxia treatment.

Secondly, high levels of oxygen leads to increases free oxygen radicals (Reactive oxygen species) formation in tumor cells. This ROS causes “oxidative stress” resulting into the cell death via apoptosis. Oxidative stress occurs only when free oxygen species concentration exceeds endogenous anti-oxidant cellular concentration. In normal cells, superoxide dismutase (SOD) concentration that counteracts ROS is more as compared to the tumor cells, thereby
affecting tumor cells in high order. Though ROS causes DNA damage in normal cells it induces apoptic cell death in tumors [18].

1.4. Nanotechnology for Tumor-Targeted and Intracellular Drug Delivery

Although chemotherapy is highly preferred way to treat the cancer, the treatment approach has several disadvantages in terms of pharmacokinetic profile. The intravenous administration of the drugs accounts for the poor bio distribution profile, rapid clearance from the body, inefficient targeting property and toxicity on the healthy cells. These all disadvantages resulted into the development of targeted nanomedicine. Tumor targeted nanomedicine are the drug delivery systems that overcome the above disadvantages of the chemotherapeutic drugs. Main feature of this systems are *site specific and site avoidance delivery* of the drugs. This can be achieved either by *passive targeting* or *active targeting* of the drug delivery systems. Former is achieved because of the leaky vasculature and enhanced permeability and retention effect (EPR) of the tumor cells, while latter by conjugating nano carriers with peptides, polymers, ligands and antibodies specific to particular tumors [19, 20]. However, thorough understanding of the basic biological profile is required for the development of the tumor targeted drug delivery systems.

1.4.1a. Passive and Active Targeted Delivery Systems: Most of the tumors are characterized by presence of leaky vasculature and poor lymphatic drainage system. Long circulating nano carries take advantage of this property of tumor by extravasating through leaky vessels in to interstitial fluid of tumor. In addition, poor lymphatic drainage retains the nano carriers at tumor site for prolong period for time thereby increasing efficacy of the drug. This property of the tumor is also known as *enhanced permeability and retention effect* [19, 21].

**Figure 11** shows the process of passive tumor targeting.
Various drug delivery systems used which are widely used are polymeric nanoparticles, liposomes and micelles, dendrimers. Polymeric drug conjugates (nano particles) are nano carriers in which drugs are conjugated to the side chain of polymer via linker which is susceptible to cleavage under specific conditions. Natural polymer such as albumin, gelatin and chitosan are used to form drug conjugates. Abraxane has been used in the clinical trials of metastatic breast cancer, in which albumin is conjugated with paclitaxel. Also, synthetic polymers such as polyethylene glycol (PEG), N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) and poly (L-glutamic acid) (PGA) are widely used. Xyotax, a PGA-paclitaxel nanoparticle system are being studied in clinical trials [20, 21]. Also, modified PEG polymers such have been developed to use as a nanocarriers [22]. Liposomes are the self assembling lipid bilayer that can encapsulate either hydrophilic or hydrophobic drugs. They are highly versatile form of nano carriers with biodegradable and biocompatible properties. Doxil, liposomal formulation of doxorubicin has been approved to treat metastatic breast cancer [21]. However, all the tumors do not exhibit EPR leading to change in the permeability rate of nanocarriers. Also their efficacy decreases in due to development of multiple-drug resistance.
To overcome the disadvantages of passive drug targeting, concept of specific drug targeting was developed. Here specific ligands or antibodies are attached to polymer drug conjugate systems that bind specifically to receptors or antigens expressed on tumor cells. Factors such as antigen or receptor expression and internalization are taken into consideration for the development of active targeted systems. Antigen and receptors must be homogenous and over expressed only in tumor cells and they must not be present in the blood and must be specific to different types of cancer [21, 23]. Figure 12 shows receptor mediated endocytosis of targeted nano particles. This is achieved via targeting nanoparticles to carbohydrates, receptors and antigens present on the tumor cells. Carbohydrate-directed targeting involves the development of nanoparticles coated either by carbohydrates (direct lectin targeting) or by lectins itself (reverse lectin targeting). Lectins are the binding protein that plays an important role in tumor metastasis by interacting with surface carbohydrates present on tumor cells. Receptor or antigen mediated targeting involves the attachment of specific ligand or antibodies onto the surface of drug-polymer conjugate. Internalization of the drugs occurs via receptor mediated endocytosis as it occurs in normal cells [23].

Various agents are used to develop tumor targeted drug delivery systems such as

Figure 12: Mechanism of active tumor targeting through receptor-mediated endocytosis.
antibodies, aptamers, and ligands such as vitamins, peptides or carbohydrates. Antibodies are either used as a whole or in fragments for targeting purpose. But, complete monoclonal antibodies are suitable because of high binding due to presence of two binding fragments. Bevacizumab (Avastin®) an anti-VEGF monoclonal antibody is used in angiogenesis, Trastuzumab (Herceptin®) an anti HER-2 monoclonal antibody binds to ErbB2 receptor, used to treat in breast cancer [23]. Also antibodies can be engineered to increase their binding affinity towards specific confirmation of target receptor. Aptamers are short single stranded DNA or RNA that binds to the intracellular, transmembrane proteins, carbohydrates and small molecule drugs. Docetaxel loaded with aptamers modified nanoparticles are used to target specific antigen present on the prostate cancer cells [23]. Also, vitamins and other growth factor are attached to the polymer drug conjugate systems for active tumor targeting.

1.5. Rationale for Combination of Chemo and Hyperoxia Therapy

Despite the increasing advanced in development of targeted nano-therapeutics, some amount of non-specific drug uptake by the normal cells still persist, which leads to unwanted side effects and ultimately non-compliance with the treatment. Also, the use of combination of multiple chemotherapeutic drugs in the treatment of cancer is not effective in completely removing the root cause in the particular cancer. So, there is a critical need to use another parallel treatment which can affect the core biology of the cancer and can prevent the relapse of cancer. Thus, the idea of using hyperoxia, increased levels of oxygen, was introduced along with the use of chemotherapeutic therapy. The reason behind the use of hyperoxia as a treatment is to reverse the natural hypoxia-induced effects in tumor cells. Along with this treatment, chemotherapeutic agent doxorubicin was used as a second line of therapy.
Doxorubicin (DOX) is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius* var. *caesius*. It consists of a naphthacenequinine nucleus linked through a glycosidic bond at ring atom 7 to duanosamine sugar. The anthracycline nucleus intercalates with DNA double helix which inhibits nucleotide replication and action of DNA and RNA polymerases. Also interaction with topoisomerase II forms a complex which cleaves the DNA and binding to cell membrane and various plasma proteins leads to activation of apoptosis cascade. However, enzymatic reduction of doxorubicin generates highly reactive oxygen species which has been implicated in doxorubicin cardio toxicity. Here, in order to incorporate in to the hydrophobic core of oil in water nano emulsion, a hydrophobic DOX derivative, DOX stearate, which was prepared by salt formation between DOX hydrochloride and sodium stearate. Extra pure grade fish oil was used as an oil phase as it contains omega 3 fatty acids, which can act as tumor suppressor agents in breast cancer by inducing modification in the biosynthesis of eicosanoids from n-6 poly unsaturated fatty acids [24, 25]. Egg phosphotidylcholine, if used, provides integrity and stabilizes the system. Surface modification can be done with PEG as it will decrease the uptake by RES without affecting the pharmacokinetic profile and temperature sensitivity of the lipid.

The standard treatment of breast cancer involves surgical removal followed by post-operative tamoxifen-based chemotherapy. So, use of the hyperoxia reverses the effect of hypoxia inducible factor-1 (HIF-1) and thereby other factors involved in the proliferation, metastasis and angiogenesis. Also due to the vasodilatation of the central and peripheral vessel, tumor blood flow increases leading to the increase in the delivery of doxorubicin stearate at the tumor site. DOX, therefore, is used which exerts is effect by totally different mechanism. Although no
clinical trials exist which evaluate the effect of hyperoxia in breast cancer, several groups have used combination of hyperoxia and chemotherapy and have got encouraging results [26].

In order to evaluate these hypotheses, in our study, we decided to evaluate the effect of hyperoxia along with doxorubicin stearate encapsulated in oil-in-water nanoemulsions on MDA-MB-231 triple negative human breast adenocarcinoma cells.
2. OBJECTIVES AND SPECIFIC AIMS

2.1. Statement of the Problem

Cancer is second leading cause of deaths accounting for 12.5% of the total death that occurs in the world. Also, the worldwide estimated new cancer cases in 2007 were about 12.3 million out of which more than 2.0 million were estimated in the United States [1]. One of the major problems in cancer therapy is the inefficient systemic delivery of the chemotherapeutic agents into the tumor area. A conventional chemotherapeutic strategy involves the systemic administration of the chemotherapeutic agents which gets wide distributed in to the various regions of the body leading to poor efficacy and systemic toxicity. Moreover, these agents are not effectively delivered in to the tumor area because of poor perfusion, hence unable to treat root cause due to lower residence time. Current chemotherapeutic strategies enhances efficacy of these agents via administration of combination of different chemotherapeutic agents having different mechanisms to treat cancer cells. Also, the dose of each agent was increased up to a maximum tolerated dose. Another challenge in the delivery of chemotherapeutic agents to tumor mass in vivo is poor perfusion into specific regions that lack adequate blood supply and are hypoxic. The tumor microenvironment around the tumor creates regions of hypoxia due to poor oxygen perfusion. Combination of poor drug delivery efficiency and micro environmental selection pressure is known to contribute to tumor drug resistance and aggressiveness.

2.2. Objectives and Experimental Hypotheses

Nanotechnology-based drug delivery systems have shown to enhance the delivery efficiency in cancer therapy by increasing drug accumulation in the tumor mass, decreasing non-specific accumulation in other organs of the body, and mitigating the toxicity of chemotherapeutic agents via passive and active targeting mechanism. However, this
conventional nanocarrier system alone are not efficient in delivering chemotherapeutic agents into the tumor region core because of high degree of irregularity in blood vessels and hypoxic condition prevailing in the tumor area. Combination of nanocarrier-based drug delivery and hyperoxia can have a profound impact on enhancing the efficacy of chemotherapeutic agents by reversing the hypoxia induced activity of HIF systems. Tumor cells have been found to be highly hypoxic and so hyperoxia were lead to increased oxygen concentration into the tumor core thereby decreasing HIF activity. Also, increased oxygen were lead to decrease angiogenesis and improving tumor blood flow which enhances delivery of chemotherapeutic agent into the tumor mass. Thus, hyperoxia therapy will have a synergistic effect in the treatment of cancer.

To examine the effect of combination hyperoxia and drug delivery in cancer, in this study, we developed and evaluate oil-in-water (O/W) nanoemulsion formulations that can encapsulate hydrophobic anticancer drugs. Oil rich in omega 3 poly unsaturated fatty acid was selected to prepare O/W nanoemulsion based on the previous studies conducted in our lab using these types of oils with chemotherapeutic agent. The O/W nanoemulsions are versatile delivery system based on the selection of single or combination therapeutic agents, type of oil used, and the choice of surfactants for stabilization of the nano-sized oil droplets. In vitro studies were performed to evaluate the intracellular accumulation of drugs using drug loaded nanoemulsion and the enhancement in cell-kill efficacy and apoptotic response when drug and hyperoxia are combined.

Our experimental hypotheses for the studies proposed are as follows: (1) stable formulation of O/W nanoemulsion formulated to release the encapsulated drug (2) increasing the oxygen were enhance drug accumulation in tumor cells when administered with nanoemulsion
and (3) the combination of drug and hyperoxia therapy were have a synergistic effect in enhancing killing of tumor cell

2.3. Specific Aims

The specific aims that were evaluated in this MS dissertation project are:

Aim 1: Formulation and Characterization of Doxorubicin Stearate– Encapsulating Oil-in-Water Nanoemulsion Formulation Systems

a) Synthesis and characterization of doxorubicin stearate (DOX stearate) salt using doxorubicin hydrochloride and sodium stearate.
b) Preparation of blank and drug-loaded O/W nanoemulsions using omega-3 fatty acid rich fish oil as the internal oil phase.
c) Encapsulation of DOX stearate in the nanoemulsion and characterization for size, surface charge, particle morphology (by TEM), and the drug encapsulation efficiency.

Aim 2: Drug Uptake Studies and Reactive Oxygen Species Level Detection under Different Incubation Conditions:

a) Establishment and maintenance of MDA-MB-231 human breast adenocarcinoma cells in culture.
b) Qualitative Uptake studies of DOX Stearate in solution or in nanoemulsion by confocal microscopy in MDA-MB-231 human breast cancer cell line under hypoxia, normoxia and hyperoxia conditions.
c) Quantitative Uptake studies of DOX Stearate in solution or in nanoemulsion by confocal microscopy in MDA-MB-231 human breast cancer cell line under hypoxia, normoxia and hyperoxia conditions.
d) Determination of reactive oxygen species levels in MDA-MB-231 human breast cancer cells under hypoxia, normoxia and hyperoxia.

**Aim 3: Evaluation of Cytotoxicity and Apoptotic Activity of DOX Stearate Encapsulated in Nanoemulsion and Hyperoxia Combination on MDA-MB-231 Human Breast Adenocarcinoma Cells**

a) Evaluation of cytotoxicity of DOX stearate in solution or in nanoemulsion using MTT (formazan) assay and determination of IC$_{50}$ values under hypoxia, normoxia, and hyperoxia.

b) Quantitative evaluation of apoptotic activity of DOX stearate in solution or in nanoemulsion with caspase 3/7 levels.

c) Qualitative evaluation of apoptotic activity of DOX stearate in solution or in nanoemulsion using TUNEL (DNA fragmentation) assay.

e) Qualitative and quantitative analysis of the incubation conditions (i.e., hypoxia, normoxia, and hyperoxia) on expression of hypoxia-inducing factor-alpha (HIF-1α) mRNA by RT-PCR and qPCR.

f) Qualitative analysis of the incubation conditions (i.e., hypoxia, normoxia, and hyperoxia) on expression of hypoxia-inducing factor-alpha (HIF-1α) protein by western blot.
3. EXPERIMENTAL DESIGN AND METHODS

3.1 Synthesis of Doxorubicin Stearate Complex

Doxorubicin hydrochloride (Axxora LLC, San Diego, CA) is a highly hydrophilic anticancer drug and so it cannot be encapsulated in the hydrophobic phase of the oil-in-water nanoemulsion. As such, a hydrophobic complex derivative, doxorubicin stearate (Figure 13C), was prepared by reacting doxorubicin hydrochloride with sodium stearate. Also, since stearate is a saturated fatty acid, it know to induce apoptosis in tumor cells via ceramide modulation [27]. Both, doxorubicin hydrochloride and sodium stearate were weighed according to 1:10 molar ratio and dissolved in pH 8.6 borate buffer [28]. Sodium stearate is only sparingly soluble in cold water and, therefore, the buffer was warmed and the fatty acid salt was slowly added until it completely dissolved. Both the solutions were mixed and homogenized for 10 seconds. The product formed was extracted by liquid-liquid extraction using methylene chloride as the organic phase and borate buffer as the aqueous phase. Doxorubicin stearate was partitioned into the organic phase and methylene chloride was evaporated to obtain the dry powder. $^1$H-NMR analysis was done to confirm the presence of doxorubicin stearate in the final composition. Also yield was calculated and powder was stored in a vial at 4°C. Desired stock solution was prepared using methylene chloride as a solvent.

3.2 Preparation of Blank and Drug-Loaded Nanoemulsion Formulations

In order to develop a delivery vehicle for intracellular administration of doxorubicin stearate in normoxic, hypoxic, and hyperoxic tumor cells, we formulated an oil-in-water nanoemulsion system using EPA and DHA containing extra pure fish oil (Jedwards International, Inc., Quincy, MA) as the inner hydrophobic phase and egg phosphatidylcholine...
(Lipoid-E80®) as the primary emulsifier. Both blank and drug-loaded nanoemulsions were prepared using the ultrasound method previously optimized in our lab [29, 30].

3.2.1. Blank Nanoemulsion: The aqueous phase consisted of 4 mL of de-ionized distilled water, 120 mg of egg phoshotidylcholine (Lipoid® E80), and 15 mg of MPEG2000DSPE in a 20 mL glass scintillation vial. The aqueous phase was stirred for 30 minutes on the magnetic stirrer for complete dissolution of both the components. In a separate vial, 1 gram of fish oil was carefully measured. Both the vials are heated in a water bath at 70°C for approximately 10 minutes. Subsequently, the aqueous phase was added gradually into the oil phase and the resulting mixture was ultrasonicated at 21% amplitude using the Sonics and Materials Vibra-Cell VC 505® probe ultrasound instrument (Newtown, CT) for 10 minutes. The nanoemulsion formed was transferred to a new vial and kept in a refrigerator at 4°C until further use.

3.2.2. Drug-Containing Nanoemulsion: Doxorubicin stearate was weighed accordingly to give 1 mM final concentration and dissolved in methylene chloride. The stock solution was added into a clean vial containing 1 gram of fish oil and methylene chloride was evaporated by slowly passing nitrogen gas into the vial. Doxorubicin stearate was mixed thoroughly to insure complete dissolution in the oil phase of the nanoemulsion. The aqueous phase was the same as the one used to make blank nanoemulsion. After heating at 70°C, the aqueous phase was slowly added and stirred with the oil phase and the resulting mixture was ultrasonicated at 21% amplitude using the Vibra-Cell VC 505® probe ultrasound instrument for 10 minutes. The nanoemulsions formed were transferred to a new vial covered with aluminum foil and stored in the dark at 4°C until further use.
3.3 Characterization of the Nanoemulsion Formulations

Both blank and doxorubicin stearate-loaded nanoemulsions were characterized for oil droplet size and size distribution, surface charge (zeta potential), and the morphology was evaluated using transmission electron microscopy (TEM). For the drug-loaded nanoemulsion formulation, the encapsulation efficiency of doxorubicin stearate was also determined.

3.3.1. Particle Size Analysis: Particle size and size distribution of the oil droplets in the blank and drug-containing nanoemulsion formulations were determined using the ZetaSizer (Malvern Instrument Inc., Westborough, MA). The ZetaSizer measures particle size using the principle of dynamic light scattering (DLS). About 10 µl of the nanoemulsion sample was diluted to 10 ml with deionized distilled water and the oil droplet size was determined at 90° angle and at 25°C temperature. The average count rate was kept below 1 megaCPS. The mean number diameter of the oil droplets was obtained on log-normal distribution mode. Both the mean droplet diameter and the polydispersity index was measured and reported.

3.3.2. Zeta Potential: The surface charge values measure the relative stability of a colloidal dispersion due to electrostatic repulsion. Zeta potential has also been correlated with cellular uptake and biological performance of nanoparticle-based delivery systems. ZetaSizer instrument (Malvern Instrument Inc., Westborough, AR) was used for measurement of zeta potential. About 10 µl of the nanoemulsion sample was diluted to 10 ml with deionized water and then placed in a specialized cuvette, which was connected to an electrode. The instrument measures the electrophoretic mobility of the oil droplets upon application of charge gradient. The electrophoretic mobility values were then converted to zeta potential using Henry’s Law equation using built-in software, which follows, the Smoluchowski’s approximation.
3.3.3. **TEM Analysis:** TEM analysis was carried out in order to evaluate the morphology of the oil droplet and confirm the size with the DLS measurements. About 50 μL of blank and doxorubicin stearate-containing nanoemulsion formulations were placed on a Formvar-coated copper grid (Electron Microscopy Sciences, Hatfield, PA) and were allowed to air-dry after draining off the excess emulsion using a Whatman filter paper. Negative staining of the samples was performed using uranyl acetate for 10 minutes at room temperature and the excess reagent was drained off using a Whatman filter paper. The resulting thin film of the nanoemulsion sample on the copper grid was observed with a JEOL 100-X transmission electron microscope (Peabody, MA).

3.3.4. **Drug Encapsulation Studies:** The encapsulation efficiency of doxorubicin stearate in the drug-containing nanoemulsion sample was determined by ultra-filtration method using Amicon® ultra centrifugal filter devices (molecular weight cut-off 3000, Millipore, Bedford, MA). The nanoemulsion sample (0.5 mL) was placed in the upper donor chamber and the unit was centrifuged at 11,000 rpm for 30 minutes. A standard curve of the drug was obtained by measuring the fluorescent intensity of five different concentration of doxorubicin hydrochloride. Then, the concentration of doxorubicin stearate in the aqueous phase was estimated by measuring the fluorescence at the excitation wavelength of 525 nm and emission wavelength of 645 nm using the Bio-Tek Instrument’s Synergy® HT (Winooski, VT) microplate reader. The percentage of doxorubicin stearate remaining in the oil phase was calculated by mass balance and was used for determine the encapsulation efficiency.
3.4 Establishment and Maintenance of MDA-MB 231 Triple Negative Human Breast Adenocarcinoma Cells in Culture

3.4.1. Cell Culture Conditions: MDA-MB-231 triple negative human breast adenocarcinoma cells obtained from ATCC (Manassas, VA), were grown in RPMI medium supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. Cell cultures were maintained in an incubator maintained at 5% CO₂ and 37°C. Sub culturing was done by detaching the monolayer from the culture flask by using 0.05% trypsin/EDTA.

3.4.2. Hypoxia and Hyperoxia Treatment. MDA-MB-231 human breast adenocarcinoma cells were placed in an incubator and exposed to gas mixtures with different ratio of nitrogen, carbon dioxide, and oxygen to simulate hypoxia, normoxia, and hyperoxia conditions in vitro [26]. Prefilled gas mixtures in tanks used for this study were supplied by MED-TECH® Gases (Medford, MA).

For hypoxia, the gas mixture with 0.5% oxygen, 5% carbon dioxide, and 94.5% nitrogen was used. For normoxic cell growth, 21% oxygen, 5% carbon dioxide, and 74% nitrogen was used. Lastly two different oxygen concentrations were used for hyperoxia simulation. As such, two different gas mixture with 40% and 60% oxygen were used having 5% carbon dioxide and 45% and 35% nitrogen, respectively. The cells were placed in T-25 flasks cultured and were incubated in Modulator Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA) and chambers were filled with respective gases for hypoxia and hyperoxia treatments. Once the gas chambers were filled with the gas mixtures, they were placed in the cell culture incubator for specific time points.
3.5 Cellular Uptake Studies using Confocal Fluorescence Microscopy

DOX stearate containing nanoemulsion and solution (1mM) was prepared for cellular uptake. DOX hydrochloride solution was used as a control. 20 µL of 1mM nanoemulsion or solutions were diluted to 2 mL with the RPMI growth medium and the cellular uptake studies were performed under hypoxia, normoxia and hyperoxia incubation conditions.

MDA MB 231 human breast adenocarcinoma cells were incubated in the culture flask, harvested by trypsinization, and seeded in 6 well- plate with cover slip in place prior to the seeding. The cell density was kept between 300,000 to 400,000 cells per well and were allowed to adhere overnight. The media was removed and replaced with 2 mL of the DOX stearate solution, DOX stearate nanoemulsion, and DOX hydrochloride solution. Plates were incubated for 6 hours at 37°C under hypoxic, normoxic and hyperoxic (40% and 60%) conditions. After 6 hours of incubation, the wells were washed twice with chilled PBS to ensure complete removal of the non-internalized emulsion or solutions. Nuclear stain was performed using Hoechst 33342 nucleic acid stain (Invitrogen, CA). Dye was diluted in ratio of 1:10000 in RPMI media and cell were incubated with 2 ml of dye solution for 5 minutes. Again cells were washed twice with chilled PBS. Then cells were fixed by adding 2 ml of cold 4% w/v paraformaldehyde (w/v) in PBS and incubated at room temperature for 25 minutes. After the fixation, cells were washed twice with cold PBS to ensure complete removal of Para formaldehyde to prevent crystal formation. Then cover slip was mounted in invert position on slide using Fluor mount-G™. Zeiss LSM 700 scanning confocal microscope was used to obtain images 40X magnification. Texas Red® filter was used for DOX stearate and DAPI for the nuclear Stain. Media and Blank nanoemulsion were used as negative control.
3.6 Quantitative Intracellular Drug Concentrations

To quantitate the intracellular concentrations, fluorescence spectroscopy and bicinchonic acid (BCA) protein assay was performed whereby the concentrations of DOX stearate in the cells were determined relative to the total amount of proteins in the cell. Standard curve for total protein, DOX hydrochloride and DOX stearate was made.

Intracellular drug uptake studies were performed with DOX hydrochloride, DOX stearate solution in dimethylsulfoxide (DMSO), and DOX stearate nanoemulsion at DOX concentrations of 0.1 µM, 1 µM and 10 µM. About 100,000 cells were seeded in the 6-well plate and would be allowed to adhere by incubating the plate overnight. The media was replaced with 2 ml of DOX hydrochloride, DOX stearate solution and nanoemulsion in media at above three concentrations. Plates were incubated for 6 hours at 37°C under hypoxic, normoxic, 40% and 60% hyperoxic conditions. After 6 hours of incubation, the wells were washed twice with 1% PBS solution to ensure complete removal of the non-internalized solution or emulsion. Cells were trypsinized and pellet obtained was subjected to cell lysis buffer (as per cell lysis protocol). The supernatant obtained was evaluated for the total amount of proteins using the BCA assay. To determine the amount of doxorubicin stearate, 100 µL of the supernatant was diluted with 100 µL of DMSO and the fluorescence intensity was measured at an excitation wavelength of 525 nm and emission wavelength of 645 nm using Bio-Tek Instrument’s Synergy® HT microplate reader. The amount of total proteins and DOX in DOX hydrochloride, DOX stearate solution and nanoemulsion in the lysate was determined by extrapolating the readings obtained from the standard curve. The final DOX stearate concentration was reported as ng of the drug per µg of total cellular protein.
3.7 Formation of Reactive Oxygen Species

To detect the reactive oxygen species levels in different incubation conditions hypoxia, normoxia and hyperoxia, detection kit from Cell Technology was used. The kit is based on principle of fluorescence spectroscopy. Hydroxyphenyl fluorescein (HPF) is the selective dye for the detection of highly reactive oxygen species. (hROS). This dye fluorescence when comes in contact with hROS (hydroxyl radical: \( \text{OH}^- \), Peroxynitrite: \( \text{ONOO}^- \)) and exhibit strong dose dependent fluorescence.

About 7000 cells were seeded per 96 well plates and each plate was incubated for 3 days under hypoxic, normoxic, 40% and 60% hyperoxic conditions. After 3 days, each plate was washed with modified HBSS. HPF dye (5 mM) was diluted at ratio of 1:10 with HBSS and 4 \( \mu l \) of the diluted dye was added per 200 \( \mu l \) of the media in each well. The final concentration of the dye in the well was 10 \( \mu M \). After the addition of the dye, plates were incubated for 60 minutes in dark at room temperature. Fluorescence was measured at excitation wavelength of 488 nm and emission wavelength of 515 nm using Bio-Tek Instrument’s Synergy® HT microplate reader.

3.8 Cytotoxicity with Combination Drug and Oxygen Therapies

DOX hydrochloride, DOX stearate solution in DMSO, and DOX stearate-loaded nanoemulsion formulations was used to determine dose-dependent growth inhibitory effect on MDA-MB-231 human breast cancer cells using MTT (formazan) assay. Various concentrations ranging from 10 nM to 20 \( \mu M \) doxorubicin equivalent was prepared and the concentration required to inhibit the cell growth by 50% (IC\textsubscript{50}) was determined using Graph Pad Prism\textsuperscript{®} 4 software. DOX hydrochloride and DOX stearate were dissolved in DMSO to obtain 1 mM stock solution and this was used for serial dilutions in the RPMI cell culture media to obtain graded final concentrations of the drug solution. DOX stearate nanoemulsion was also diluted with
RPMI to obtain the graded concentrations. Approximately, 7,000 MDA-MB-231 cells per well was seeded into a 96-well microplate and was allowed to adhere overnight. After the incubation, the media was replaced with various concentrations of drug solutions and nanoemulsion formulations. Treatment with 0.25 mg/mL poly (ethyleneimine) (molecular weight 10 kDa), a cytotoxic cationic polymer, was used as a positive control. Negative control was treatment with RPMI growth medium alone. To evaluate the effect of DMSO and emulsion components on cell viability, the highest concentration of DMSO and blank nanoemulsion was also used as controls. Eight replicates were made for each test condition and plates were incubated for 3 days under hypoxia, normoxia, 40% and 60% hyperoxia. After 3 days, plates were washed with media thrice and then media was replaced with 100 µL of MTT (1.0 mg/mL in RPMI) reagent and the plates were incubated for 4 hours at 37°C. 150 µL DMSO was added to dissolve the insoluble formazan crystals and absorbance was measured at 570 nm using Bio-Tek Instrument’s Synergy® HT microplate reader. The percentage cell viability values were determined relative to negative control and IC₅₀ values were calculated using Graph Pad Prism® 4 software.

3.9 Quantitative Apoptosis Studies by Caspase 3/7 Assay

For quantitative apoptotic analysis, MDA-MB 231 cells were seeded in 96-well microplates at a density of 20,000 cells per well. Apoptosis was induced by treating the cells with doxorubicin solution or doxorubicin stearate solution or doxorubicin stearate in nanoemulsion under hypoxic, normoxic, 40% and 60 hyperoxic conditions. The concentrations used were 0.1 µM, 1 µM and 10 µM. The cells were incubated for 6 hours at 37°C under different oxygen conditions based on the treatment group, followed by wash with RPMI to remove any drug that did not enter the cells and further incubation for 24 hours at 37°C. After this period, 100 µL of the Apo-ONE® Caspase-3/7 substrate solution was added to each well
containing 100 µL of RPMI medium. The contents of wells were mixed using a plate shaker at 300-500 rpm for 2 hours at room temperature. Fluorescence of each well was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm using a Bio-Tek Instrument’s Synergy® HT microplate reader. Caspase-3/7 activity was reported as percent activation relative to untreated control.

3.10 Qualitative Apoptosis Studies by TUNEL Assay

3.10.1 Principle: Terminal deoxynucleotidyl transferase dUTP nick end labeling, known as TUNEL assay is the method of detecting DNA fragments generated via apoptotic signal. This system, end-labels fragmented DNA with biotinylated nucleotide at the 3′OH ends using the recombinant Terminal Deoxynucleotidyl Transferase (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (streptavidin-HRP) are then bound to the biotinylated nucleotide which is detected using peroxidase substrate, hydrogen peroxide and the chromogen diaminobenzidine (DAB), which confers the apoptotic nuclei brown color. A DeadEnd™ Colorimetric TUNEL System kit from Promega Corporation (Madison, WI) was used for this study.

3.10.2. Procedure: About 100,000 cells were seeded on coverslip in the 6-well plate and would be allowed to adhere by incubating the plate overnight. Cells were then dosed with 2 ml of DOX hydrochloride solution, DOX stearate solution and nanoemulsion at concentration of 0.1 µM, 1 µM and 10 µM and incubated under hypoxia, normoxia, 40% and 60% hyperoxia for 24 hours. The cells were then washed twice with PBS and fixed by using 10% buffered formalin for 25 minutes at room temperature (RT) followed by washing twice in PBS for 5 minutes at RT. The cells were then be permeabilized using 0.2% Triton® X-100 solution in PBS for 5 minutes at RT followed by washing twice in PBS for 5 minutes each at RT. Some cells can be treated with DNAase I which exposes multiple 3′OH DNA and can be used as a positive control for detection
of DNA fragmentation. Excess liquid would be tapped off the slides, after which cells were equilibrated for 5-10 minutes with 100 μL of equilibration buffer. 100 μL rTdT reaction mix per coverslip was prepared using 98 μL of equilibration buffer + 1 μL of biotinylated nucleotide mix + 1 μL of rTdT enzyme. 100 μL of the rTdT reaction mix was added to the previously equilibrated sections on the slide without allowing sections to dry and then covered with plate lid to ensure even distribution which was incubated at 37°C, 5% CO₂ for 60 minutes. The cover slips were washed in 2X sodium chloride and sodium citrate (SSC) buffer for 15 minutes at RT. The cells were washed twice in PBS for 5 minutes at RT to remove the unincorporated biotinylated nucleotides and then immersed in 0.3% hydrogen peroxide solution in PBS at RT for 3-5 minutes to block endogenous peroxidases. Again, the cells were washed twice in PBS for 5 minutes at RT. 100 μL of diluted streptavidin-HRP (1:500 in PBS) was added per well followed by 30 minute incubation at RT. The cells were then washed twice by immersing in PBS for 5 minutes at RT. 100 μL of freshly prepared DAB solution (50 μL DAB Substrate 20X Buffer + 950 μL deionized water + 50 μL DAB 20X Chromogen + 50 μL Hydrogen Peroxide 20X) was added to the sections until a light brown color develops. The cells were then be rinsed several times in deionized water and then cover slips were mounted in permanent mounting medium and observed under a light microscope.

### 3.11 HIF-1α Expression Analysis by RT-PCR

The cells were placed in T-75 flasks cultured and were incubated in Modulator Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA) and chambers were filled with respective gases for hypoxia and hyperoxia treatments. Once the gas chambers were filled with the gas mixtures, they were placed in the cell culture incubator for specific time points (3 days).
3.11.1. RNA Extraction and Quantification: After exposure of the cells with hypoxia, normoxia, and hyperoxia treatments, the RNA samples were isolated using High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) after 3 day and 5 day for the respective treatment. All RNAs extracted were quantified using the Nano-Drop 2000 (Thermo Scientific, Wilmington, DE). The RNA samples were stored in -80°C until further use.

3.11.2. Reverse Transcriptase Polymerase Chain Reaction: RT-PCR was carried out as per the protocol mentioned in the One Step RT-PCR Kit (QIAGEN, Valencia, CA) using PCR sprint Temperature Cycling System (Thermo Scientific, Rockford, IL). RT-PCR was performed separately for the different markers like HIF 1α, VEGF, MMP-2, MMP-9 and GLUT1 each with 45 cycles. Beta-actin, used as a loading control, was run as a control for 35 cycles. The amplified products were stored at -20 °C for further use.

3.11.3. Agarose Gel Electrophoresis: The amplified products obtained were identified using 1.2% SYBR Safe® E-Gels as per the protocol. After 30 minutes run, all gels were analyzed using the Image J software for semi-quantitative analysis.

3.12 Western Blot Analysis of HIF- 1α Expression

The cells were placed in T-75 flasks cultured and were incubated in Modulator Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA) and chambers were filled with respective gases for hypoxia and hyperoxia treatments. Once the gas chambers were filled with the gas mixtures, they were placed in the cell culture incubator for specific time points (3 days). After exposure of the cells with hypoxia, normoxia, and hyperoxia treatments, protein extraction was carried out using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL, USA). Following the protocol in this kit, both nuclear and cytoplasmic protein extraction was
carried out. The total protein content was estimated using Pierce’s BCA protein quantitation assay (Rockford, IL). A standard curve was constructed using bovine serum albumin. Protein samples were kept in ice bucket, volume equivalent to 15 µg of protein was transfer to the respective labeled 1.5ml tubes on ice. Equivalent volume of Laemmli sample buffer (Bio-Rad, CA, USA) was added to each sample. All the samples were heated for 5 min at 90°C in a water bath. After heating, tubes were transferred to ice bucket and samples were spinned briefly before loading the gel. The denatured samples were loaded onto pre-cast 4-15% SDS-polyacrylamide gel electrophoresis (PAGE) gradient gel (Bio-Rad, Hercules, CA). Gel was ran for 30 minutes at 200 V using Tris/Glycine/SDS running buffer and the protein bands were transferred on the nitrocellulose membrane using the iBLOT device (Invitrogen, Carlsbad, CA). Now the membrane was blocked with 5% blotto 1X TBS-T for one hour at room temperature. After blocking, the membrane was washed thrice with 1X TBS-T for 5 minutes by rocking. Primary antibody Rabbit monoclonal to HIF1 alpha (abcam Inc., Cambridge, MA) was diluted in a ratio of 1:2000 using 1X TBS-T buffer and added on to the membrane and the membrane was rocked overnight at 4°C. After incubating membrane overnight, primary antibody was removed and membrane was washed thrice with 1x TBS-T buffer. Now, anti-rabbit IgG- HRP linked antibody (Cell signaling Technology, MA, USA) was diluted to ration of 1:10000 using 1x TBS-T buffer and added on the membrane and rocked at room temperature for 1 hour. Again, the membrane was washed thrice with 1x TBS-T buffer. Finally, luminata forte western HRP substrate (Millipore, USA) was added enough to cover the membrane and incubated in dark at room temperature for 5 minutes. Then, the membrane was covered with plastic and chemiluminescent bands were visualized using a Kodak imager (Carestream Health, Rochester, NY). Same procedure was followed for the β-actin (house keeping) antibody.
4. RESULTS AND DISCUSSION

4.1 Synthesis, Purification, and NMR Analysis of DOX Stearate Complex

DOX stearate was synthesized using the liquid – liquid extraction method. The synthesized drug was extracted out in the methylene chloride fraction of the extraction process, from which dry powder was obtained by evaporating the methylene chloride. The dry powder was analyzed using $^1$H-NMR to identify whether the product obtained has DOX and stearate part in 1:1 ratio. For this, $^1$H-NMR spectra of the parent moiety DOX hydrochloride (Figure 13A) and Sodium stearate (Figure 13B) was also obtained.

In Figure 13A the peak between $\delta=7$ and $\delta=8$ is due to the presence of the aromatic ring present in the doxorubicin moiety which is main factor indicating the presence of Doxorubicin moiety, the peak between $\delta=5.2$ and $\delta=5.4$ is due to the presence of RCH=CHR and the peak between $\delta=2.8-3$ is due to aromatic CH which is also the part of the doxorubicin moiety. In Figure 13B all the peaks obtained are at lower $\delta$ values, which are mainly due to CH=CH
present in the long chain of the stearate moiety. Now spectra of the DOX stearate, as showed in Figure 13C-D, shows the presence of the aromatic ring of the DOX moiety at higher δ same as in spectra of DOX hydrochloride. However, the intensity of the peak is far less as compared to the intensity of the peaks due to the stearate part of the product obtained. Also the intensity of the peak due to hydrogen from the other part of the DOX moiety RCH=CHR, aromatic hydrogen are also less as compared to that of hydrogen from the stearate moiety. From the values calculated with respect to the total hydrogen intensities of the DOX to that of the stearate moiety, it was confirmed that for 1 molecule of DOX there were 13 molecules of the stearate moiety. This leads to the conclusion that the product obtained is in DOX stearate complex instead of the salt.

Figure 13: $^1$H-NMR of the Doxorubicin Hydrochloride solution (A), Sodium Stearate (B) and Doxorubicin Stearate Nanoemulsion (C-D).

4.2 Preparation and Characterization of the Nanoemulsion Formulations
Omega-3 fatty acid-rich fish oil-containing oil-in-water nanoemulsions were formulated using egg phosphotidylcholine as the principle phospholipid, which helped in reducing the surface tension and MPEG-2000-DSPE which provided the stearic stability. The prepared nanoemulsion formulations were characterized with respect to the oil droplet hydrodynamic diameters, surface charge (zeta potential) values, drug encapsulation efficiency, and were observed by transmission electron microscopy (TEM).

4.2.1 Particle Size Analysis and Surface Charge Measurements

The results of particle size analysis and zeta potential measurements shown in Table 1. It shows the particle size and polydispersity index of the blank and drug-containing nanoemulsion formulations. In each case, the size ranged from 185-190 nm with a narrow distribution. Also, it shows the average zeta potential values of the blank and drug-containing nanoemulsion formulations. The zeta potential values for both the nanoemulsion preparations were found to be highly negative due to presence of egg phosphotidylcholine. Due to the presence of greater than -30 mV of zeta potential on all of the formulations, they were found to be very stable under storage.

4.2.2 Drug Encapsulation Efficiency
The result of the drug encapsulation analysis from the Table 2 shows that almost 100% of the drug that has been added during the formulation retained in the oil phase of the nanoemulsion.

Table 2: Encapsulation efficiency of doxorubicin stearate in oil-in-water nanoemulsion formulations.

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>Amount of Dox in Aqueous phase (mg)</th>
<th>% of Dox. In Aqueous phase</th>
<th>% Drug encapsulation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0022</td>
<td>0.04%</td>
<td>99.92</td>
</tr>
<tr>
<td>2</td>
<td>0.0038</td>
<td>0.14%</td>
<td>99.86</td>
</tr>
<tr>
<td>3</td>
<td>0.003</td>
<td>0.11%</td>
<td>99.89</td>
</tr>
</tbody>
</table>

4.2.3 Transmission Electron Microscopy (TEM) Analysis

The TEM images in Figure 14 shows the surface morphology of the oil droplets in blank and drug-encapsulated nanoemulsion formulations negatively stained with uranyl acetate.

Figure 14: Transmission electron microscopy images of the (A) Blank and (B) Doxorubicin stearate encapsulated nanoemulsion formulations negatively stained with uranyl acetate
The oil droplets in both nanoemulsion samples were spherical in shape and were homogenously distributed.

### 4.3 Qualitative Cellular Drug Uptake Studies by Confocal Microscopy

After the successful formulation of the DOX stearate nanoemulsion based on the characterization studies above, qualitative uptake study of the DOX stearate solution and nanoemulsion was performed.

**Figure 15:** Confocal microscopy images showing the cellular uptake of Doxorubicin Hydrochloride, Doxorubicin Stearate solution and Doxorubicin Stearate nanoemulsion in MDA MB 231 cells followed by 6 hrs of incubation. Panel’s A-E-I, B-F-J, C-G-K and D-H-L
DOX hydrochloride solution was used as a control to compare the uptake of DOX stearate solution and nanoemulsion. Cells were incubated with this treatment under hypoxia, normoxia, 40% hyperoxia and 60% hyperoxia for 6 hours. Figure 15 shows the confocal microscopy images of the uptake of the DOX Hydrochloride solution, DOX stearate solution and nanoemulsion under different oxygen conditions. Figure 15 (A-D) shows uptake of the DOX Hydrochloride solution, Figure 15 (E-H) shows the uptake of DOX stearate solution and Figure 15 (I-L) shows the uptake of DOX stearate nanoemulsion under hypoxia, normoxia, 40% and 60% hyperoxia respectively. From these images it was revealed that the DOX hydrochloride was readily available to the nucleus within 6 hrs of incubation period as compared to DOX stearate solution and nanoemulsion irrespective of the oxygen conditions. More cytoplasmic accumulation of the DOX stearate solution and nanoemulsion can be due to the endosomal uptake of this moiety. This causes the breakdown of the DOX stearate in to the DOX and stearate by the endosomal enzymes, from which DOX slowly gets release and enters into the nucleus. Thus, non-specific endosomal uptake of DOX stearate in solution and nanoemulsion can act reservoir for the DOX moiety which slowly release the DOX. However, DOX stearate nanoemulsion is readily up taken by the cells as compared to the DOX stearate solution. Moreover, we do not see the increase in the drug uptake as the concentration of the oxygen increases because the intensity remains the same across the different oxygen conditions. But, most importantly, 40% and 60% hyperoxia causes increase in the cell death as compared to the hypoxia and normoxic cells as seen from the shrinking of the cells in higher % of oxygen. This might be due to the increased amount of the reactive oxygen species which leads to the apoptosis.

4.4 Quantitative Intracellular Cellular Drug Accumulation Studies
To support the results of the qualitative drug uptake studies, Intracellular drug uptake study was performed to quantitate the amount of the drug internalized in different oxygen conditions. So, cells were treated with DOX stearate in solution and in nanoemulsion in three different concentrations under hypoxia, normoxia, 40% and 60% hyperoxia. DOX hydrochloride was taken as a comparative control. **Figure 16** showed increased uptake for the DOX stearate nanoemulsion as compared to DOX stearate solution, which supports con-focal data. However, increased levels of Doxorubicin hydrochloride, as compared to that of DOX stearate in solution and in nanoemulsion under different oxygen conditions, explains the endosomal entrapment of the DOX stearate. This endosomal entrapment might act as a reservoir for the DOX which slowly release free DOX over the period of time. Also, it is seen that hyperoxia does not aid to increase the uptake of drugs as the amount internalized remains same, which is quite surprising.

**Figure 16:** Intracellular Drug uptake studies carried out in combination of Dox stearate and oxygen therapy. A-D shows the levels of the drug uptake in MDA MB 231 cells under hypoxia, normoxia, 40% and 60% hyperoxia.
4.5 Detection of Reactive Oxygen Species Levels upon Incubation Conditions

The results obtained from qualitative drug uptake study showed the rounding of the cells under 40% and 60% hyperoxic conditions. The reason might be due to the increase production of the reactive oxygen species in these cells. To confirm this hypothesis, reactive oxygen species detection assay was performed. This assay uses specific dye that fluoresces in presence of the reactive oxygen species in dose dependent manner. In this assay, hydroxyphenyl fluorescein (HPF) dye was used which reacts with hydroxyl radical and peroxynitrite radical.

![ROS Detection Assay at 10 µM Dye Concentration](chart)

**Figure 17**: Reactive oxygen species detection assay to determine the levels of highly reactive oxygen species under different oxygen incubation conditions.

*** indicates p < 0.0001 when compared with levels in hypoxia and normoxia

**Figure 17** shows the level of the reactive oxygen species based on the fluorescent intensity at 10 µM dye concentration. From the chart it is evident that levels of reactive oxygen species (ROS) are comparatively higher in case of 40 and 60% hyperoxia as to that of hypoxia and normoxia. Significant difference exists between the levels of ROS as the percent of the
oxygen increases from hypoxia to hyperoxia based on p values obtained. This data supports the fact that hyperoxia helps in elevation of cell death via increased level of ROS.

4.6 Cytotoxicity Analyses with Combination Drug and Oxygen Therapies

Based on the results of the quantitative drug uptake and reactive oxygen species detection assay, cytotoxicity studies were carried out under different oxygen incubation conditions. The results are shown Figure 18. For example, Figure 18(A-D) shows the graph of the percent relative cell viability and IC50 values of the DOX hydrochloride solution, DOX stearate solution and DOX stearate nanoemulsion under hypoxic, normoxic, 40% and 60% hyperoxic conditions. The IC50 values of doxorubicin stearate nanoemulsion were less as compared to doxorubicin stearate solution, indicating doxorubicin stearate nanoemulsion is more cytotoxic in all four oxygen conditions, which supports above uptake data. This may be due to enhanced uptake and decreased efflux by the P-gp efflux pumps.
However, IC$_{50}$ values of the DOX stearate solution and nanoemulsion are significantly different from DOX hydrochloride in hypoxia, normoxia, 40% and 60% hyperoxia. This might be due to less availability of the DOX into the nucleus due to the endosomal uptake which also supports our uptake studies. Important thing to note about is that IC$_{50}$ values of the DOX hydrochloride, DOX stearate in solution and nanoemulsion decrease as the % of oxygen increases. However, the IC$_{50}$ values reach almost the same in 60% hyperoxia and up to certain extent in 40% hyperoxia. This can be due to gradual increase in the reactive oxygen species as the oxygen content increases in the cells. This supports the data obtained in the reactive oxygen species detection

**Figure 18:** Cytotoxicity studies carried out in combination of Dox stearate and oxygen therapy. A-D shows the cell viability graph and IC$_{50}$ values of the drugs under hypoxia, normoxia, 40% and 60% hyperoxia. * indicates p < 0.01 when compared with IC$_{50}$ values of Dox hydrochloride within the treatment.
assay. Thus, in hyperoxia, the difference is noted with a 5 fold decrease in the IC\textsubscript{50} value in case of DOX stearate nanoemulsion. This confirms our hypothesis, that hyperoxia synergistically helps in cell killing induced by DOX stearate nanoemulsion even it fails to increase the amount of the drug up taken across the different oxygen conditions.

4.7 Quantitative Apoptotic Analysis by Caspase- 3/7 Assay

Caspase -3 and -7 are members of the cysteine aspartic acid-specific protease (caspase) family and play a key effector role in apoptosis in mammalian cells. An increase in the caspase levels is indicative of the initiation of the apoptosis. The results are shown in Figure 19, which shows the caspase-3/7 activities upon treatment with doxorubicin solution, doxorubicin stearate in solution and in nanoemulsion relative to the untreated group.

From the graphs, it is evident that when hyperoxia is imparted along with the DOX stearate nanoemulsion, there is greater increase in the caspase -3/7 activity as compared to doxorubicin stearate solution within the treatment group. Also, increase in the caspase 3/7 activity is concentration dependent under hypoxia and normoxia. However, no significant difference exists between the levels of caspase 3/7 induced by the DOX hydrochloride as compared to that induced by DOX stearate nanoemulsion at 40% and 60% hyperoxia and also the increase in the caspase 3/7 activity is concentration independent at higher oxygen levels. These results collaborate with the cell viability data to confirm the synergistic effect of hyperoxia on cell-kill efficacy using DOX Stearate nanoemulsion formulation.
**Figure 19**: Caspase-3/7 activation, relative to untreated cells, in MDAMB 231 cells following treatment with doxorubicin solution (Dox), doxorubicin stearate solution (Dox St), doxorubicin stearate in nanoemulsion in hypoxia, normoxia, 40% and 60% hyperoxia (A-D). The results represent mean (n = 3) along with standard deviation.

* p < 0.05 when compared with Dox. Hydrochloride within the treatment; ** p<0.01 when compared with Dox. Hydrochloride within the treatment, ***p<0.001 when compared with Dox. Hydrochloride within the treatment, # = non significant

### 4.8 Qualitative Apoptosis Study by TUNEL Assay

TUNEL assay provides a qualitative analysis of cellular apoptosis. Drug treatment leading to apoptosis will result in DNA fragmentation causing the apoptotic nuclei to be stained brown by a three-step mechanism that involves incorporation of biotinylated nucleotides at 3′-OH DNA end, binding of horseradish peroxidase-labeled streptavidin to the incorporated nucleotides, and finally the detection of the nucleotides with the help of peroxidase substrate,
diaminobenzidine, resulting in the formation of the dark brown stains. As shown in the Figure 20, the untreated cells (media) do not show brown colored nuclei suggesting no apoptosis.

![TUNEL staining images of untreated MDA MB 231 cells in the normoxic conditions](image)

**Figure 20:** TUNEL staining images of untreated MDA MB 231 cells in the normoxic conditions

The cells treated with the blank nanoemulsions developed a faint brown color (Figure 20) indicating mild toxicity. The cells treated with DOX Hydrochloride, DOX stearate and DOX Stearate nanoemulsion as shown in Figure 21-24 under hypoxia, normoxia, 40% and 60% hyperoxia showed the characteristic brown colored nuclei, indicating the apoptotic cells. However, the cells treated with DOX hydrochloride and DOX stearate nanoemulsions showed highest degree of apoptosis which could be inferred from a more pronounced dark brown colored nuclei as compared to the DOX stearate solution which supports all of its above data.
Figure 21: TUNEL staining images of treated MDA MB 231 cells in the Hypoxic conditions

Figure 22: TUNEL staining images of treated MDA MB 231 cells in the Normoxic conditions
The assay was also carried out in presence of three different concentrations along with the oxygen treatment. In this case, higher degree of apoptosis was observed in all the treatment types at higher concentrations, but still, it was highest in cells treated with 40% and 60% hyperoxia even at low concentrations of DOX hydrochloride and DOX stearate nanoemulsion. These results were found to be in correlation with the ROS study, cytotoxicity and Caspase results suggesting the synergistic effect of hyperoxia co-administered with DOX stearate nanoemulsion formulation in MDA-MB-231 cells.

Figure 23: TUNEL staining images of treated MDA MB 231 cells in the 40% Hyperoxic conditions
RT-PCR analysis result as shown in Figure 25 reveals that no changes in the expression of the HIF-1α was observed at the mRNA level under different oxygen incubation conditions. Also, semi quantitative analysis (not showed), using NIH Image J software, of the HIF-1α bands showed no changes in the expression irrespective of the oxygen conditions. This can be explained in a way that oxygen increases the expression of HIF-1α post-transcriptional. Secondly, this might be due to in house flaw in RT –PCR assay requiring HIF-1α specific primers.
4.10 qPCR Analysis of HIF-1α Expression at mRNA Levels

To overcome the problems that might have occurred in RT PCR analysis, real time PCR was done using HIF-1α specific q-PCR array kit. However, as shown in Figure 26, contradictory results were obtained having high relative expression of the HIF-1α in the hyperoxia as compared to hypoxia. Whether there exists significant difference between relative expression of HIF-1α in hyperoxia and hypoxia is yet to be determined. Slight Increase in the HIF-1α in the 40 and 60% Hyperoxia also needs to be explained. Although HIF-1α specific primers have been used changes in HIF levels were not detected. This might be a method problem and needs to be repeated.
Figure 26: Determination of relative expression of the HIF-1α at mRNA level under different oxygen conditions using HIF-1α specific qPCR array.

4.11 Western Blot Analysis of HIF-1α Protein Expression

Protein expression of the in HIF-1α in Figure 27 shows promising but surprising results as the expression is very less in hypoxia which is contradictory to current literatures. However effect of the oxygen has been observed as HIF-1α protein gets degraded as % oxygen increases from normoxia to 40% to 60% hyperoxia. The reason for very light band (Hypoxia) is might be due to improper binding of primary Ab or it can be problem of hypoxia protein extract as we can see B Actin band is also of less thickness as compared to other. Also, it might be due to improper nuclear extraction which leads to more cytoplasmic extract. If this is the case then, for hypoxia HIF-1α has already been enter the nucleus and so less amount will be present in the cytoplasmic extract. However this needs to be repeated again in a better way with proper nuclear house keeping protein.
Figure 27: Down-regulation of HIF-1α by Western blot analysis.

- 15 μg of protein was loaded in each lane.
- Lane 1: Hypoxia
- Lane 2: Normoxia
- Lane 3: 40% Hyperoxia
- Lane 4: 60% Hyperoxia
5. CONCLUSIONS

The result of this study has shown that combination of chemo- and hyperoxia therapies has significant potential for the treatment of cancer, even multidrug resistant form. Nanoemulsions prepared using ultrasound method and fish oil, rich in omega-3 PUFA, could efficiently encapsulate the hydrophobic form of the drug and thus was found to be a suitable carrier. The qualitative microscopy study showed rapid uptake of the DOX stearate nanoemulsion but gets entrap in the endosomes which acts as a reservoir for the release of free DOX over period of time. This finding was supported by the quantitative intracellular drug accumulation data. Cellular uptake and distribution studies reveal that the nanoemulsions were internalized by the cells indicating their possible role as an effective delivery system. Moreover, microscopy study showed increase cell killing in hyperoxia treatment which was proved from the increased levels of reactive oxygen species. The cytotoxicity results demonstrate that the drug encapsulated in nanoemulsions had lower IC50 value as compared to free drug in solution indicating their higher efficiency in killing cells. Synergistic effect was observed even in the resistant cell line when chemotherapy was combined with hyperoxia as a treatment modality. The combination therapy resulted in the high degree of apoptosis which is evident from the qualitative TUNEL assay and quantitative caspase-3/7. The results of western blot analysis have shown promising results of the effect of hyperoxia on the degradation of the HIF-1α; however more in-depth and proper molecular biology studies needs to be done to prove this. Alone, hyperoxia has shown promising results in decreasing the aggressiveness of cancer as evident from cytotoxicity, caspase 3/7 and TUNEL assay. Thus, the combinational therapy of chemo and hyperoxia has significant potential in clinical management of breast cancer. Further in vivo studies are required in order to validate these findings.
6. REFERENCES


