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The Effect of Target-Specific Biomolecules in Breast Cancer

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THE EFFECT OF TARGET-SPECIFIC BIOMOLECULES IN BREAST CANCER

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Mohannad Mrwan Garoub

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To: Dean Tomás R. Guilarte
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This dissertation, written by Mohannad Mrwan Garoub, and entitled The Effect of Target-Specific Biomolecules in Breast Cancer, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

I dedicate my dissertation work to my wonderful family. A special feeling of gratitude to my loving parents, Mrwan and Saleha, whose words of encouragement and inspiration ring in my ears. My sisters Fatimah, Mrwah, and Luijain and my brothers Mohammed, Hussain, and Abdulmalik have never left my side and supported me all the way through my dissertation. Also, my loving and understanding wife, Sara, who has patiently put up with these many years of research with unconditional love and care.

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ABSTRACT OF THE DISSERTATION

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Professor Marcus S. Cooke, Co-Major Professor

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Cancer is the second leading cause of mortality in the United States and the World, therefore, early effective prevention, diagnosis, and therapy is needed. Estrogens play a major role in the initiation and progression of breast cancer. Elevated lifetime exposure to estrogens is associated with an increased risk of developing breast cancer. Estrogens through influencing mitochondria contribute to estrogen induced breast carcinogenesis; however, the exact mitochondrial mechanisms underlying the estrogen carcinogenic effect in breast tissue are not clearly understood. For this dissertation, the mitotoxic and cytotoxic effects of triphenylphosphonium cation (TPP) and *Origanum majorana* organic extract (OME) as well as PEGylated bioconjugate of OME with TPP (P-OME-TPP) against human breast epithelial and cancer cell lines was investigated. Initially, TPP, a lipophilic cation, was used to check whether an imbalance in mitochondrial bioenergetics, in part, may be responsible for estrogen induced growth of breast cancer. The results showed that exposure of estrogen-dependent MCF-7 cells to 17 β-estradiol (E2) induced the metabolic activity, proliferation, mitochondrial bioenergetics, DNA damage, and formation of cellular and

mitochondrial reactive oxidant species (ROS). These E2-induced endpoints were inhibited by co-treatment with TPP, indicating mitochondrial mechanisms, in part, may contribute to the development of breast cancer. Furthermore, *O. majorana*, widely used in the Middle East as a culinary aromatic medicinal herb, has been shown to possess an extensive range of biological activity including antioxidant, anti-inflammatory, and anti-tumor growth effects. Interestingly, the anticancer potential of *O. majorana* against breast cancer remains largely unexplored; therefore, the anticancer effect of *O. majorana* on breast cell lines was investigated. The results showed that E2-induced metabolic activity and growth were inhibited by OME in MCF-7 cells. The results also demonstrated that synthesized P-OME-TPP conjugate, compared to OME, was far more effective in exerting its cytotoxic effect through the inhibition of growth and mitochondrial metabolic activity in both highly metastatic, triple negative MDA-MB-231 and estrogen-dependent MCF-7 breast cancer cells. Altogether, these findings offer a new perspective on the utility of mitochondria-targeted lipophilic TPP cation and the potential of *O. majorana* extract to be developed as a new therapy against breast tumors.

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LIST OF ABBREVIATIONS

BCSC	Breast cancer stem cell
CSC	Cancer stem cell
DCFDA	2′,7′-Dichlorofluorescin diacetate
DMSO	Dimethylsulfoxide
DOX	Doxorubicin
E2	17β-estradiol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FBS	Fetal bovine serum
HER2	Human epidermal growth factor receptor 2
MRC	Mitochondrial respiratory chain
MRI	Magnetic resonance imaging
mt ROS	Mitochondrial ROS
mTOR	Mammalian target of rapamycin
MTT	3(4,5-dimethylthiazolyl-2)2,5-diphenyl tetrazolium bromide
NIR	Near infrared
NRF1	Nuclear respiratory factor 1
O. majorana	*Origanum majorana*
OME	*O. majorana* organic extract
P123	Pluronic 123
PARP	Poly ADP-ribose polymerase
PEG	Polyethylene glycol
PGC-1α	Coactivator 1α
PI	Propidium iodide
P-OME-TPP	Bioconjugate of OME with PEG and TPP
PR	Progesterone receptor

PTEN	Phosphatase and tensin homolog
RES	Reticuloendothelial system
Rh123	Rhodamine
ROS	Reactive oxygen species
SERM	Selective estrogen receptor modulator
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TNBC	Triple-negative breast cancer
TPP	Triphenylphosphonium
uPA	Urokinase-type plasminogen activator
UPR^{mt}	Mitochondrial unfolded protein response
VEGF	Vascular endothelial growth factor
$\Delta\Psi_m$	Mitochondrial membrane potential
$\Delta\Psi_p$	Plasma membrane potential
RT	Room temperature

CHAPTER 1

1.1 INTRODUCTION

Breast cancer is the most common cancer in women and the second most common cancer among all cancers worldwide, and in the United States.[1] In 2017, almost 30% (255,180 out of 852,630 cases) of newly diagnosed cases and 14% (41,070 out of 282,500 cases) of cancer-related deaths are expected breast cancer cases among all cancer sites in female, in the USA.[1] These high rates are probably due to the interaction of several environmental and biological factors. These interactions, genetically and epigenetically, make breast cancer a heterogeneous disease, which leads to therapeutic challenges including resistance and recurrence, and the need for extremely toxic therapeutic options. Indeed, almost half of breast cancer patients are treated with hormonal therapy and chemotherapy; however, 35% of those patients are resistant to this therapy.[2] Treatments against aggressive tumors, usually resistant to therapy, are not effective. The existing therapeutic agents used on aggressive tumors, such as chemotherapy, are extremely toxic.[3] There is an urgent need to develop more effective and less toxic therapy against aggressive breast cancer. To achieve this, it is necessary to develop therapies based on the clinical and molecular characteristics of the tumor. Based on gene expression profiles, there are at least three significant subtypes.[4] Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) receptor status are very important in categorizing breast tumors to successfully predict outcome and help in treating the disease.[5]

For years, estrogen was suspected to play major role in cancer, since strong epidemiological and clinical evidence linked exposure to estrogens with increased risk of

developing breast, endometrial, and uterine cancers.[6–8] Elevated lifetime exposure of estrogen is caused by early menstruating and/or late-onset menopause; however, the exact routes of estrogens take in promoting tumor development are not clearly understood. The National Institute of Environmental Health Sciences added estrogen to its list of known cancer-causing agents, due to its capability to induce and promote the development of malignant neoplasms.[9–12] In addition, breast cancer characterized by lack of ER, PR, and HER2, often called triple-negative breast cancer (TNBC), is typically associated with poor prognosis due to aggressive tumor phenotypes and current lack of effective and specific therapies.[13] Traditional chemotherapy drugs cause non-specific and toxic off-target effects on normal tissues, deteriorate the patient's quality of life, and weaken the immune system.[14]

Mitochondria are considered important bioenergetic and biosynthetic factories that play a critical role in cellular metabolism, calcium homeostasis, redox signaling, apoptosis and cell death.[15] Tumor cells are characterized by dysfunctional mitochondria, which is demonstrated by the mitochondrial respiration defects that shift the source of metabolic energy from oxidative phosphorylation to active glycolysis and increase reactive oxygen species (ROS) production.[16] Estrogens have been shown to increase mitochondria-derived ROS (mt ROS) that might contribute to breast carcinogenesis, although the exact role of mitochondria in the carcinogenic effect is unclear.[17] One of the few proposed mechanisms suggested that E2-induced mt ROS can act as signal transducing messengers by activating the binding of oxidant-sensitive transcription factors known to be involved in the regulation of cell cycle genes.[18] Moreover, specific mitochondrial activities can contribute to the initiation and progression of tumor growth.[19] For instance, mechanisms involved in cancer

cell metabolic reprogramming have been shown to be associated with oncogenic signals.[20] In recent years, mitochondria have been recognized as a potential therapeutic target in cancer therapy, a concept that has emerged and is an acknowledged area of reaseach.[21]

Mitochondria-specific accumulation of therapeutic agents is necessary in optimizing the therapeutic efficacy of tumor-targeted drugs. A broad applicable approach in mitochondria-specific delivery is using triphenylphosphonium (TPP) cation to deliver various bioactive molecules to mitochondria including antioxidants.[22] TPP is a lipophilic cation, which allows it to pass through the phospholipid bilayers of the cell membrane and mitochondria.[23] The large mitochondrial membrane potential, approximately -180 mV, across the membrane layers allows the uptake and accumulation of TPP in mitochondria.[23]

An increasing number of studies have shown evidence that phytochemicals are important with regards to their cancer chemoprevention properties and their ability to decrease tumor growth.[24] Phytochemicals can regulate several pathways used by cancer cells in the processes of cell growth and proliferation, survival, angiogenesis, invasion, metastasis, and apoptosis.[25] *Origanum majorana*, commonly known as marjoram, is worldwide perennial herb. It is utilized as a spice, flavoring agent, and has an ancient culinary and medicinal use. It has been used as traditional medicine for various illnesses such as chest infections, cough, sore throat, rheumatism, cardiovascular diseases, stomach ache, as well as skin care. Several studies have reported that *O. majorana* is rich in phenolic compounds, which possess the capacity to scavenge free radicals and exhibit strong antioxidant properties.[26] A few studies have reported the anticancer potential of *O. majorana*; however, it remains largely unexplored.[27]

To increase drugs efficacy and lower their toxicity, small molecule bioconjugation therapy could be the solution, since it has the ability to specifically target and safely reach tumor core, due to its unique physical and biological properties.[28] Polyethylene glycol (PEG), is an amphiphilic polymer, which provides electrostatic and steric stabilization, and a longer circulation half-life *in vivo* as well as functional-end groups for the attachment of targeting ligands such as antibodies, peptides and aptamers.[28] PEGylation of anticancer molecules is to protect the molecules from destruction by the reticuloendothelial system (RES) and, therefore, increase circulation time and drug accumulation in the tumors.[29] Thus, developing target specific PEG conjugate with *O. majorana* and TPP as therapeutic molecules against breast cancer cells is a rational approach for breast cancer therapy. The novel concept of this study was a bioconjugation approach that involves PEGylation of natural anti-tumor compounds and specific mitochondria targeting molecules for specifically preventing growth of breast cancer and possibly other types of cancers.

1.2 HYPOTHESIS AND SPECIFIC AIMS

It is hypothesized that novel mitochondria-targeted bioconjugates of *O. majorana* organic extract (OME) with polyethylene glycol (PEG) and triphenylphosphonium (TPP), inhibits the growth of breast cancer cells.

In order to test this hypothesis, the following specific aims have been identified:

Aim 1. To test the effect of the mitochondria-targeted lipophilic cation TPP, E2, and co-treatment of E2 with TPP in estrogen-dependent breast cancer (MCF-7), metastatic breast cancer (MDA-MB231), and normal epithelial breast (MCF-10A) cells. The purpose of this

aim is to elucidate the functions by which mitochondria contribute to the growth of breast cancer. Cell viability, cell growth, and cell proliferation assays were used to study the cytotoxic effect of TPP and its E2 co-treatment. Mitochondrial bioenergetics, cellular and mitochondrial ROS production, and DNA damage assays were used to identify potential molecular effect of TPP, E2, and co-treatment of E2 with TPP.

Aim 2. To synthesize and test a small molecule bioconjugate of OME with PEG and TPP (P-OME-TPP). The purpose of this aim is to test the potential cytotoxic effects of OME as well as P-OME-TPP against MCF-7, MDA-MB213, and MCF-10A cell lines in the presence and absence of E2. Cell viability and cell growth assays were used to study the cytotoxic effectiveness of OME and P-OME-TPP. The findings of this study will determine the possible antitumor properties of OME and P-OME-TPP as a potential breast cancer therapy.

CHAPTER 2

LITERATURE REVIEW

CURRENT STATUS OF TARGETING BREAST CANCER STEM CELLS USING SMALL MOLECULE-BASED THERAPY FOR TRIPLE NEGATIVE AGGRESSIVE BREAST CANCER

2.1 Abstract

Breast cancer is the most common malignancies among women and about 20% of this disease is represented by triple negative breast carcinoma (TNBC). Triple-negative breast cancer, characterized by tumors lacking expression of estrogen receptor, progesterone receptor, or human epidermal growth factor 2 amplification, impose a clinical challenge because they are often resistant to conventional therapy. Due to the absence of well-defined molecular targets, the aggressive nature of TNBC, poor prognosis, and low efficacy and high toxicity of existing therapeutic agents, identification of prognostic factors and markers to identify a novel treatment approach is utmost needed therapy for this disease. Cancer stem cells (CSCs) are predicted to mediate tumor recurrence after therapy due to their properties of self-renewal and differentiation. Tumor resistance to therapy is linked to both CSCs and dysfunctional mitochondria. Recently, targeted therapeutic approaches have been utilized in breast cancer and other types of cancer. Developing target-specific therapy for CSCs signaling pathways and mitochondrial functions could be a possible approach for TNBC. In this sense, nanoparticle-based therapy is capable of combining various targeted molecules that specifically and safely reach the tumor with enhanced efficacy and low toxicity. This review focuses on the characterization and function of CSCs and their role in breast cancer, potential roles of several molecular markers in aggressive breast cancer stem cells (BCSCs), and mitochondrial role in cancer and CSCs as a potential target for therapy, as well as various potential nano-therapy approaches for breast cancer.

2.2 Introduction

Breast cancer is the most frequently diagnosed cancer in women and the second most common cancer among all cancers worldwide and in the United States.[1] In 2017, more than 255,000 new cases of breast cancer are expected in the USA, with almost 42,000 cancer-related deaths (Table 2.2.1).[1] The probable cause of these high rates could be the interaction of multiple environmental and biological factors. These interactions contribute to the heterogeneity of the disease, which lead to therapeutic challenges such as disease resistance and recurrence, and the need of highly toxic therapy.[2] Treatments against aggressive breast tumor are usually not effective due to disease resistance to existing therapy such as chemotherapy, which is usually immensely toxic.[3] There is a necessity of developing an effective and less toxic therapy against aggressive breast cancer. It is necessary to establish therapies according to the molecular characteristics of the tumor to achieve a better treatment with increased efficacy and low toxicity.

Breast cancer; biologically and molecularly, is classified into different subtypes, which play a significant role in the prognosis and treatment of the diseases.[4] There are at least three significant subtypes have been identified based on gene expression profiles, which include estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER-2) receptor.[5] The status of these gene expression profiles is essential in subcategorizing breast tumors for better outcome prediction and help in controlling disease progression and developing targeted treatment.[6]

Estrogens are considered a major risk factor for breast cancer initiation and progression. Epidemiological and clinical evidence have linked elevated lifetime exposure

	Estimated New Cases			Estimated Deaths		
Breast Cancer	Male	Female	Both sexes	Male	Female	Both sexes
	2,470	252,710	255,180	460	40,610	41,070
Percentage*	30%			14%		

*Percentages of all sites (100%) in female; new cases (852,630); deaths (282,500).

Table 2.2.1 Estimated Number and Percentage of New Breast Cancer Cases and Deaths. The estimated incidence and mortality data are sorted by sex and reported by American Cancer Society. Data are representative in the US populations for year 2017.[1]

to estrogen with high risk of developing breast cancer.[7–9] However, the exact mechanisms of estrogens carcinogenic effect involvement in breast tissue are still not widely explored. Since they possess the ability of initiating and promoting the development of breast malignancy, estrogens are considered, experimentally, breast carcinogens.[10–13] ER signaling pathways are one of the most identified pathways in estrogen carcinogenic mechanisms.[14,15] As most types of hormonal therapies for breast cancer are for ER+ and/or PR+ cancers, they are generally limited to anti-estrogenic and/or anti-progesteronic agents targeting their receptors.[16] Although, 70% of newly diagnosed breast cancers patients are ER+, up to 50% of those patients develop resistance to most of the hormonal therapeutic agents.[17] Furthermore, breast cancers with overexpression of HER-2 (HER-2-positive), which is overexpressed on the cancer cell surface of almost 20% of breast cancer patients,[6] tend to grow and spread more aggressively than HER-2-negative.[18] HER-2 gene amplification, which is associated with HER-2 protein overexpression in breast cancer, has been linked with promotion of tumorigenesis such as increased cell proliferation, tumor invasiveness, aggressive metastases, higher angiogenesis, and declined apoptosis.[19] Several drugs have been developed to target ER and the receptor of HER-2 protein to inhibit pathways that overexpress the epidermal growth factor (EGF) receptors in the cell membrane.[20] However, patients which lack ER and overexpress HER-2 are unresponsive to these types of therapies.[21]

The last course of action when hormone therapy (ER+) and protein targeted therapy (HER-2-positive) prove unresponsive is usually chemotherapy. Chemotherapy is mainly used for patients with advanced breast cancer, where the cancer has developed resistance

to conventional therapy and distant metastasis. For early breast cancer stages, some common chemotherapy drugs used in treatment include anthracyclines; such as Doxorubicin and Epirubicin, and taxanes; such as Paclitaxel and Docetaxel.[22] For advanced breast cancer stages, a combination of drugs are used including Docetaxel, Paclitaxel, Platinum agents, Vinorelbine, Capecitabine, Liposomal doxorubicin, Gemcitabine and more.[22] The potency of these chemotherapy drugs on *in vitro* cancer cells is clearly demonstrated at even nanomolar levels.[23] However, due to their non-specific effects in the body on normal tissues, these drugs cause toxicity, deteriorate the patient's quality of life, weaken the host immune system, and result in a severe damage to human's own recovery power.[23] Their effective mechanisms are usually on cells that actively growing and dividing quickly, which is why they work against rapidly dividing and growing cancer cells. However, many normal cells in the body, such those in the bone marrow, mucosal cells in the mouth, lining of the intestines, and hair follicles, also divide rapidly, which are the most likely off-target cells to be affected by these drugs and can lead to very toxic side effects including death.[22] Radiation therapy and/or surgery could be involved in the treatment of breast cancer and also could be combined with chemotherapy for certain advanced/late stages of breast cancer.[24]

Breast cancer characterized by lack of ER+, PR+, and HER-2-positive, often called TNBC, is usually associated with poor prognosis due to aggressive tumor phenotypes and current lack of effective and specific therapies and poor response to toxic chemotherapy.[25] TNBC contains stem cells of different origins and each one of which may not be killed by only one specific therapeutic agent. CSCs possess several characteristics including self-

renewal, pluripotency and tumorigenicity and form a rare population in a tumor tissue (11-30 percent in breast tumor).[26] Because this small population of CSCs is thought to be more resistance to chemotherapy than the major tumor cells and conventional cancer therapies do not eradicate these cells, and responsible for tumor relapse and metastasis.[26] Therefore, CSCs need to be specifically targeted and destroyed to reach total tumor elimination, a concept that has started to change approaches to cancer therapy treatment (Figure 2.2.1).[27]

Specific mitochondria activities have been linked to the initiation and progression of tumor growth.[28] Dysfunctional mitochondria is common among several cancer cells and mechanisms involved in cancer cell metabolic changes have been associated with oncogenic signals.[27] This has attracted attention of multiple research and clinical investigations on mitochondria-targeting as a cancer therapeutic approach. Mitochondrial targeting could be achieved through a specific carrier that mediates multiple delivery molecules including mitochondrial apoptotic agents or chemicals. Nanotechnology-based therapy has the ability to specifically target and reach the tumor with increased efficacy and low toxicity due to their unique physical and biological properties.[23] At present, nanomedicine is considered a new medical science with rapid growth and development, and its nanotherapy approaches can be diagnostic or therapeutic.[23] Nanocarriers like liposomes and micelles offer a non-chemical approach to modify the disposition of drug molecules.[28] For instance, liposomes and nanoparticles can be modified with antibodies or other targeting ligands to achieve cell-specific recognition.[29] Nanocarriers could be the ultimate tool in mitochondria-targeted anticancer approaches, since they not only achieve tumor-specific accumulation of a drug but also mediate mitochondria-specific

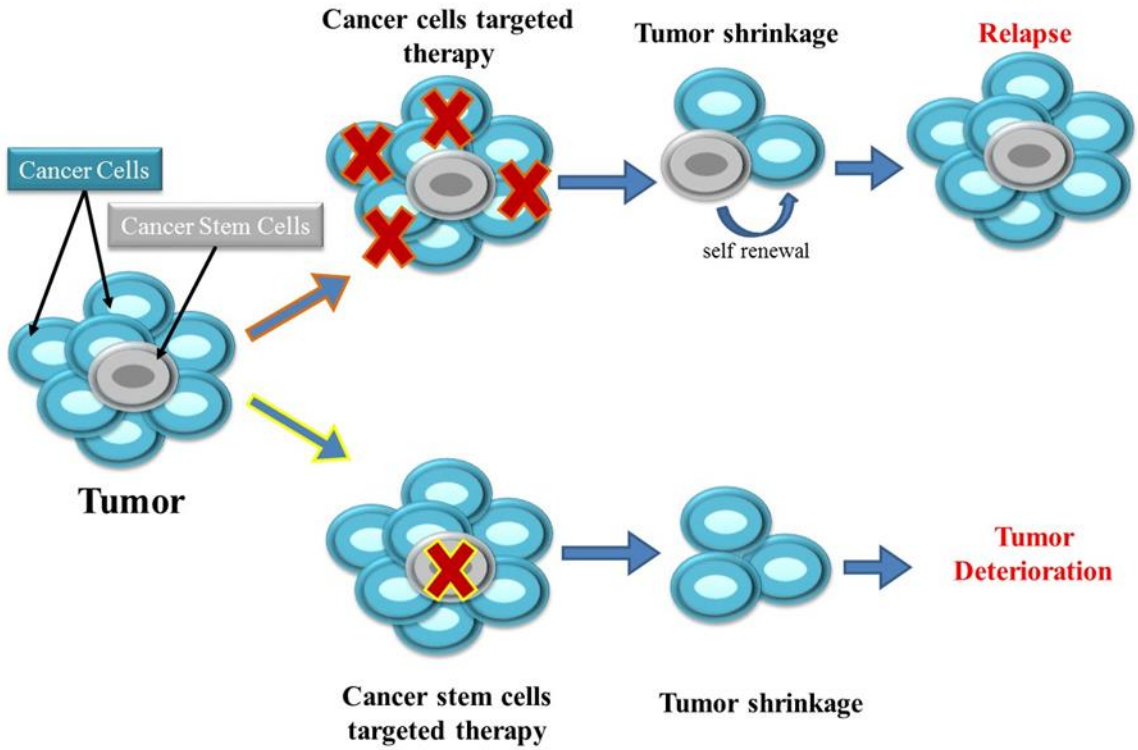


Figure 2.2.1 Therapy Targeting Cancer Stem Cells is Effective in Total Tumor Elimination. Cancer cells targeted therapy might be able to reduce the tumor size and development, but due to CSCs self-renewal property, tumor usually grow back and metastasize. In CSCs targeted therapy, the tumor usually reduces and deteriorates due to the absence of self-renewal property.

accumulation within a tumor cell.[29] In recent years, targeted therapy approaches took over conventional therapies in breast cancer and in many other cancers. As a therapy for TNBC is very challenging and difficult due to the absence of specific targets and markers, developing target-specific nano-therapeutic molecules against mitochondria and its regulated genes and proteins in TNBC and TNBC stem cells is a promising approach for TNBC therapy. In this review, CSCs and BCSCs, some molecular markers for TNBC, mitochondrial targeting in cancer and cancer stem cells, nanoparticles applications and approaches in breast cancer and BCSCs are discussed in details.

2.3 Cancer Stem Cells & Breast Cancer Stem Cells

Cancer development involves several steps and processes where healthy and normal cells undergo several stages of mutation that change them to abnormal cells that grow uncontrollably. Part of the multistep process to cancer includes acquiring damage and mutations to genes that normally regulate cell proliferation (Figure 2.3.1).[30] Accumulation of damage in these genes can result in uncontrolled cell proliferation, which can lead cells to break away from the primary tumor and form cancers at other sites in the body, a process called metastasis.[31] There is a growing body of evidence that supports the concept that CSCs are capable of both tumor initiation and sustaining tumor growth.[32] It is believed as a result from the sequential and progressive accumulation of genetic instability, adult stem cells appear to be an appropriate initial target for malignant transformation due to their long lifespans during tumor development.[32] CSCs share several properties with normal stem cells, such as capacity for self-renewal and ability to differentiate.[32] Recently, there have been extended efforts focused on identifying molecules that control the generation and cells

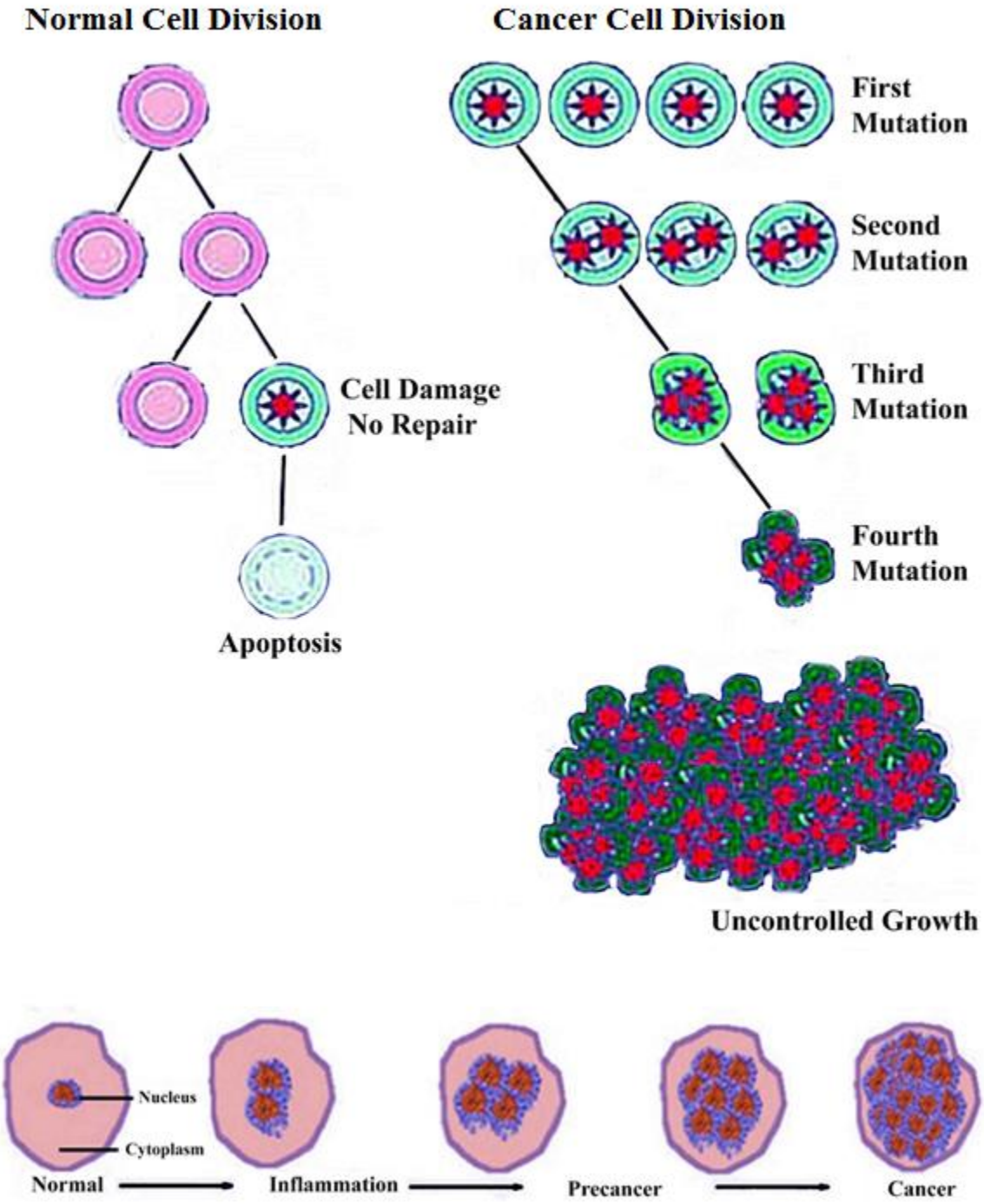


Figure 2.3.1 Emergence of Cancer Cells. Normal cell division result in many copies of cells and some copies may have accumulated DNA damage. Repair mechanisms of the cell may repair the damage or the cell may undergo apoptosis. In cancer, mutations are carried on for several cell divisions, which eventually result in uncontrollable growth and cancer. Normal cells may have some nuclear mutations which will lead to inflammation, precancer and finally cancer due to the uncontrolled cell division or growth.[33]

survival of CSCs. For identifying CSCs from tumor cells, markers specific for normal stem cells of the same organ are commonly used. Indeed, number of cell surface markers has been effectively used in the identification of CSCs including CD133, CD44, CD24, EpCAM, THY1, ATP-binding cassette B5, and CD200.[34] Specifically, BCSCs have been identified by the enrichment of two subpopulations including $CD44^{+}/CD24^{-/low}$ and $ALDH^{+}$.[34]

CD44 is an adhesion molecule with multiple isoforms that has pleiotropic roles in signaling, migration and homeostasis.[35] In 2003, Al-Hajj *et al.* made the initial discovery of BCSCs when they revealed a cellular population from human breast cancer tumors characterized by the cell-surface markers $CD44^{+}/CD24^{-/low}$.[34] They found that the $CD44^{+}/CD24^{-/low}$ subpopulation in tumors was highly tumorigenic. They obtained $CD44^{+}/CD24^{-/low}$ cells from a primary site or metastatic pleural effusions, around 1000 cells, gave rise to tumors when xenotransplanted into NOD/SCID mice.[34] In addition, CD44 is a fusogenic factor that implies that CSCs may have the capacity to fuse with other cell types, which explains the detection of fusogenic proteins commonly associated with neoplastic malignances.[36]

ALDH activity has been identified, and widely used, as a CSC marker for a variety of malignancies, including lung, liver, bone, colon, pancreatic, prostate, head and neck, bladder, thyroid, brain, melanoma and cervical cancers.[37–47] Ginestier *et al.* reported that breast cancer cells with self-renewal and differentiation properties and high tumorigenic activity were $ALDH^{+}$.[48] They were able to xenotransplant $ALDH^{+}$ cells from human breast cancer tumors into NOD/SCID mice and consecutively passaged them *in vivo*, whereas

$ALDH^-$ cells were unable to do so.[48] Cells with ALDH activity isolated from normal human breast contain mammary stem cells. Tumors induced by $ALDH^+$ cells show some phenotypic and functional characteristics similar to the original tumor, as well as tumor size and latent period correspond to the number of $ALDH^+$ cells xenotransplanted. The combination of $ALDH^+$ and $CD44^+/CD24^{-/low}$ phenotypes appeared to be highly enriched in tumorigenic capability with the ability to generate tumors from as few as 20 cells.[48]

Since BCSCs have the ability to self-renew and the potential to differentiate, they can generate cells with a variety of phenotypes within tumors.[35] Several pathways have been implicated in the regulation of BCSC self-renewal, including Notch, Hedgehog, and Wnt pathways.[35] Furthermore, key transcription factors play an important role in regulating BCSCs include NF-κB, c-Jun, Forkhead-like-protein Dach1, and CDK inhibitor p21CIP1.[35] Indeed, *in vitro* and *in vivo* evidence revealed the importance of PTEN/PI3-K/Akt/Wnt/β-catenin pathway in BCSCs biology. For instance, knocking-down PTEN in breast cancer cell lines induced activation of Akt and increased mammosphere formation and the ALDH+ population.[49] By xenotransplanting these cells into NOD/SCID mice, it increased Akt phosphorylation and tumorigenicity, which indicates that Akt is involved in regulating BCSCs expansion by activating the Akt phosphorylates GSK3β and thereby the Wnt pathway.[49] In addition, BCSCs self-renewal maintenance was shown to be regulated by the ongoing autocrine signaling via the Wnt pathway.[50]

Another important feature of BCSCs is their ability to differentiate into non-stem breast cancer cells.[48] Indeed, retinoic acid, which is oxidized from retinol by ALDH, plays a role in the control of self-renewal vs. differentiation of BCSCs.[51] ATRA, an inducer of

retinoid signaling, decreases mammosphere formation and either induces genes expressed in differentiated breast cancer cells or dowregulates several programs involved in BCSCs self-renewal such as the polycomb EZH2 network, Wnt signaling, and Akt/β-catenin signaling.[48] These findings suggest that ALDH activity and retinoid signaling regulates the BCSCs population by promoting differentiation and highlights the possible therapeutic application of compounds such as ATRA or others that force the differentiation of BCSCs.[35]

2.4 Potential Markers of Aggressive Breast Cancer Stem Cells

TNBC, lacking ER and PR expression and HER-2 amplification, poses several therapeutic challenges because of the diversity and heterogeneity of the disease and the absence of distinct molecular targets.[5] Characteristically, TNBC tumors are apparently larger in size and biologically more aggressive than other breast tumors.[25] TNBCs represent about 20% of all breast cancers, which more likely affect younger women, and prevalently diagnosed in African-American women compared to Caucasian women.[25] TNBC patients have a poorer prognosis and high rates of metastasis, relapse, and distant recurrence than patients with other breast cancer subtypes.[26] The 5-years survival rate in metastatic TNBC patients is less than 30%, and almost all patients die of their disease regardless of chemotherapy regimen, which is the first-line therapy for treatment of TNBC tumors.[27] Therefore, efforts should be focused on targeting molecular markers involved in the disease recurrence and poor prognosis to achieve a better treatment with increased efficacy and low toxicity.

One of the first perception in TNBCs was the remark that they are likely to develop in BRCA1 mutation carriers and have gene expression profile matching those of *BRCA1*-deficient tumors.[52] *BRCA1* has a critical role in DNA double strand break repair, which involves in the stability of DNA.[53] Also, for proper processing and repair of DNA breaks, poly ADP-ribose polymerase (PARP) enzymes are essential in repairing DNA damage and maintain genomic stability.[54] For instance, clinical trials in TNBC using DNA-damaging agents and PARP inhibitors show promising result in *BRCA1* mutated tumors.[55] Other identified molecular markers; such as such as VEGF, EGFR, NF-κB, mTOR, and NRF-1 contributed to the design of targeted therapeutic strategy investigating potential therapies for BCSC.

2.4.1 A potential role for *BRCA1* in breast cancer stem cells

BRCA1 is an important susceptibility gene for breast cancer, which accords substantial risks of breast cancer, mainly in the pre-menopausal age group. Classically, *BRCA1* mutation carriers develop breast tumors that grow rapidly and are high grade and estrogen receptor negative.[52] Indeed, biological and molecular features of human *BRCA1* mutation carriers suggest that one of the key functions of this gene is to act as a stem cell regulator.[56] *BRCA1* is considered as a link between breast development and breast cancer, and mutations within this gene may significantly alter both processes.[56] Additionally, several studies have demonstrated that *BRCA1* plays a critical role in mammary differentiation of stem cells.[57–59] Indeed, knocking-down *BRCA1* in primary breast epithelial cells causes an increase in cells exhibiting the stem cell marker ALDH1.[60] It is

suggested that damage and/or loss of *BRCA1* may cause an increase in genetically unstable breast stem cells.[60]

BRCA1 is important for DNA double-strand break repair by homologous recombination, and significant mutations in this gene may contribute to tumorigenesis of breast cancer.[61] In addition, PARP is one of the enzymes involved in base excision repair, a major pathway in the repair of DNA single-strand breaks.[54] Indeed, *BRCA1* dysfunction overwhelmingly signals cells to inhibit PARP enzymatic activity, which leads to the accumulation of DNA lesions. This eventually causes chromosomal instability, cell cycle arrest, and consequent apoptosis.[62] Thus, the targeted inhibition of specific DNA repair pathways, using PARP inhibitors, in aggressive breast cancer stem cells may allow the design of specific and less toxic therapies for *BRCA1*-mutant breast tumors.

2.4.2 A potential role for VEGF in breast cancer stem cells

Angiogenesis plays an essential role in the development, invasion, and metastasis of aggressive metastatic cancers, such as TNBC.[63] There are multiple angiogenic factors commonly overexpressed by aggressive and invasive breast cancers, such as vascular endothelial growth factor (VEGF).[64] VEGF is considered the most potent endothelial cell mitogen and a regulator of vascular permeability.[63] Several retrospective studies significantly associated VEGF levels with overall survival of patients with early stage breast cancer. Tumors with elevated levels of VEGF have a higher risk of recurrence than low-angiogenic tumors.[63] Nakopoulou et al. reported that aggressive breast cancer tissue strongly overexpresses VEGF receptors, specifically VEGFR-2.[65] Recently, VEGF, in addition to angiogenic effects, has been shown to possibly drive BCSC self-renewal via

VEGFR-2 and increase ALDH activity in TNBC.[66] Wang et al. suggested that VEGF could be an indicator of malignant transformation, tumorigenesis and progression of aggressive breast cancer stem cells; which usually associated with tumor differentiation.[67] Therefore, the targeted inhibition of angiogenesis, using anti-VEGF treatment, may improve the efficacy of therapeutic strategy against BCSC.

2.4.3 A potential role for EGFR in breast cancer stem cells

Epidermal growth factor receptor (EGFR) expression has been linked to breast cancer, with overexpression in more than 50% of TNBC tumors.[68] Even though EGFR amplification is rare in invasive breast cancer, TNBC tumors have a rather high frequency of EGFR amplification, approximately 25% of TNBC cases.[69] A common variant of EGFR, EGFR variant III, is a naturally occurring deletion mutant of the EGFR and predominantly active variant initially identified in a high percentage of glioblastoma multiforme brain tumors.[70] However, Del Vecchio et al. found that EGFR variant III is expressed in aggressive breast tumors and interestingly involved in the stem cells self-renewal pathway, through the Wnt pathway, of this aggressive breast cancer.[71] Moreover, a recent study has found a significant positive correlation between EGFR and tumor stem cell markers CD44/CD24 expression in patients with aggressive breast cancer.[72] Together, these findings suggest that EGFR could be a promising prognostic biomarker for TNBC. In addition, EGFR-targeted therapy, using anti-EGFR-based treatment, might have a promising therapeutic significance in TNBC.

2.4.4 A potential role for NF-κB in breast cancer stem cells

A common feature established in most breast cancer tumors is the essential activation of NF-κB, a family of transcription factors that play critical roles in cell survival, proliferation, inflammation and immunity, together with the initiation and progression of breast cancer.[73] Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) also ultimately activates NF-κB and most likely contributes to the enhanced activity of this transcription factor in aggressive breast cancer cells.[74] Moreover, NF-κB regulates the expression of anti-apoptotic genes and activates different pro-inflammatory cytokines and chemokines, which is a key molecular link between inflammation and oncogenesis initiation and progression.[74] In addition, an interesting study has shown that a temporary activation of the kinase onco-protein Src in normal breast cells have resulted in phenotypic transformation that includes the formation of multiple foci, which has the ability to form colonies in soft agar and tumors in xenografts as well as mammosphere formation.[75] This epigenetic switch, defined when a stable cell type changes into another stable cell type without any modification in DNA sequences, involves a rapid inflammation response that requires NF-κB.[75] This distinct finding confirms the key role NF-κB plays in the self-renewal capability of aggressive BCSCs, as demonstrated by Src, an oncogenic kinase that promotes the development of BCSCs and the well-defined role of NF-κB in this process. Thus, NF-κB seems to be a critical molecular marker for aggressive BCSCs, and an interesting potential target in the treatment of aggressive breast tumors.

2.4.5 A potential role for mTOR in breast cancer stem cells

Mammalian target of rapamycin (mTOR) kinase mainly controls G1 cell cycle protein synthesis, preceding cell replication.[76] It is a member of the PIKK-related kinase family, which regulates signal transduction paths linking proliferative stimuli with cell cycle progression and also mediated PI3K/AKT signaling pathway, potentially overactive in a variety of breast cancer settings.[77] A recent study has found that TNBCs exhibited a significant decrease in oxygen consumption and a substantial increase in glucose uptake and lactate production compared to receptor-positive cells.[78] It also showed that the mTOR pathway is important in regulating oxidative phosphorylation in breast cancer cells and manipulation of expression of this key molecule could significantly alter mitochondrial respiration and glucose metabolism.[78] Indeed, the PI3K/mTOR signaling pathway plays a significant role in cancer stem cells. For instance, activation of the PI3K/mTOR pathway in adult blood cells through phosphatase and tensin homolog (PTEN) serves as a negative regulator of mTOR; deletion led to the generation of leukemia-initiating cells.[79] More importantly, activation of PI3K/mTOR signaling, by knocking down PTEN, increased BCSCs.[49] Therefore, these findings suggest that mTOR could be a possible marker for TNBC and selectively targeted for inhibiting cancer stem cells for improved treatment of aggressive breast cancer.

2.4.6 A potential role for NRF1 in stem cells

Nuclear respiratory factor 1 (NRF1) plays an important role as a regulator of cell cycle genes. NRF1 binding site has been identified by comparing genome-wide locations in genes involved in DNA replication, mitosis, and cytokinesis.[80] Some of the genes which

contain NRF1 binding sites on their promoters include Cdc2, guanine-nucleotide exchange factor, RCC1, DNA polymerase-α, ornithine decarboxylase, GADD153, growth-arrest and DNA-damage-inducible protein153.[80] Mammary carcinogenesis is characterized by an increased expression of NRF1, which may be linked, with increased energy demand by rapidly proliferating cells.[81] However, NRF1's role in breast carcinogenesis may be more than mere energy modulators.[81] NRF1 has been reported to be over-expressed in a number of malignant tissues including breast cancer tissues where its levels are higher in disease regions compared to adjacent normal tissue or unaffected regions suggesting that NRF1 may play a role in breast carcinogenesis.[82] NRF1 may also play major roles in modulating cell proliferation in a manner not related to mitochondria biogenesis.[83] Up-regulating the expression of key regulators of mitochondrial biogenesis, such as a peroxisome proliferator-activated receptor γ, coactivator 1α (PGC-1α) and the transcription factor NRF1 shifts the metabolism to oxidation and suppresses fatty acid biosynthesis by energy homeostasis modulator in TNBCs.[84] A recent report has identified a regulatory branch of the mitochondrial unfolded protein response (UPR^{mt}), which is mediated by the interplay of SIRT7, a histone deacetylase, and NRF1 are coupled to cellular energy metabolism and proliferation.[85] NRF1 knockdown reduces SIRT7 expression and occupancy. While inactivation of SIRT7 causes increased mitochondrial protein folding stress, and compromised regenerative capacity of hematopoietic stem cells.[85]

Most studies on the control of mitochondrial gene expression implicate PGC-1α as a “master controller” of mitochondrial biogenesis, co-activating PPARα and NRF-1.[86] An interesting study has induced the expression of PGC-1α, PPARα, and NRF-1 during

cardiomyocyte differentiation of murine stem cells and found that the application of cardiomyocyte differentiation inducer has increased the expression of PGC-1α, PPARα, and NRF-1 coincidently in early differentiation and the increase was dose-dependently up-regulated by the inducer.[87] The parallel increase in NRF-1 ensured the coordinate induction of mtDNA transcription and replication, subsequently leading to the enhanced expression of mitochondrial proteins that are vital for respiratory chain function.[88] Also, the elevation of PGC-1α, PPARα, and NRF-1 due to the phosphorylation of the p38 MAPK, stimulated by the inducer, has a significant part in the mechanisms of the murine embryonic stem cells cardiac differentiation.[87] Altogether, NRF1 could be a novel molecular marker for stem cell targeting and more specifically an interesting molecular signature for TNBC stem cells.

Clearly, there is a major need to develop effective treatment based on molecular markers of TNBC by understanding the molecular basis of this aggressive type of breast cancer. To understand the complexity of the disease and identify the molecular markers that can be potentially targeted for therapy, more extensive biological, molecular, and genomic analysis are necessary. To provide a great perception on the heterogeneity of this disease and provide platforms on preclinical effective treatment development, we need to identify the diverse subtypes of TNBC and their molecular modulators in corresponding physiological mechanisms.

2.5 Mitochondria in Cancer and Cancer Stem Cells: A Promising Target for Therapy

Mitochondria are double membraned, made of phospholipid double layers and proteins, organelles that considered to be the main generators of ATP, metabolites for the

construction of macromolecules and reactive oxygen species (ROS).[89] Beside their role as the powerhouses of the cell, they also play a critical role in calcium homeostasis, redox signaling and cell fates.[89] Specific metabolic activities can contribute to the initiation and progression of tumor growth. (Figure 2.5.1).[89] For instance, several cancer cells are characterized by their dysfunctional mitochondria, which demonstrated by the transformation of energy metabolism from oxidative phosphorylation to active glycolysis and increased generation of ROS. These metabolic changes, are often associated with upregulation of NADPH oxidase, result in oxidative burst which characterized by the rapid production and release of ROS.[89] Importantly, mechanisms involved in cancer cell metabolic reprogramming are associated with oncogenic signals, which has recently attracted attention of multiple basic research and clinical oncology investigations on targeting mitochondria as a cancer therapeutic strategy.[90] The transformation of CSCs into a differentiated tumor is a multifactorial process which includes metabolic changes in energy conversion with increased expression of genes involved in glycolysis and alteration in Krebs's cycle flux.[91] As stem cells show a preference for anaerobic metabolism, it has been established that cancer cells activate glycolytic signal pathways for energy supply and support the connection between energy remodeling and mitochondrial apoptosis resistant.[92] These metabolic features probably reveal some similarities between CSCs and differentiated cancer cells, with mitochondria having a key role in the maintenance of self-renewal and stemness.[93] Since, mitochondria are the cell principal site of energy metabolism, the main source of ATP, and are critically involved in apoptotic cell death, mitochondrial dysfunction significantly disrupts normal cellular activities, which is a feature of several tumor characteristics.[94] Recent hypothesis suggest that altered function

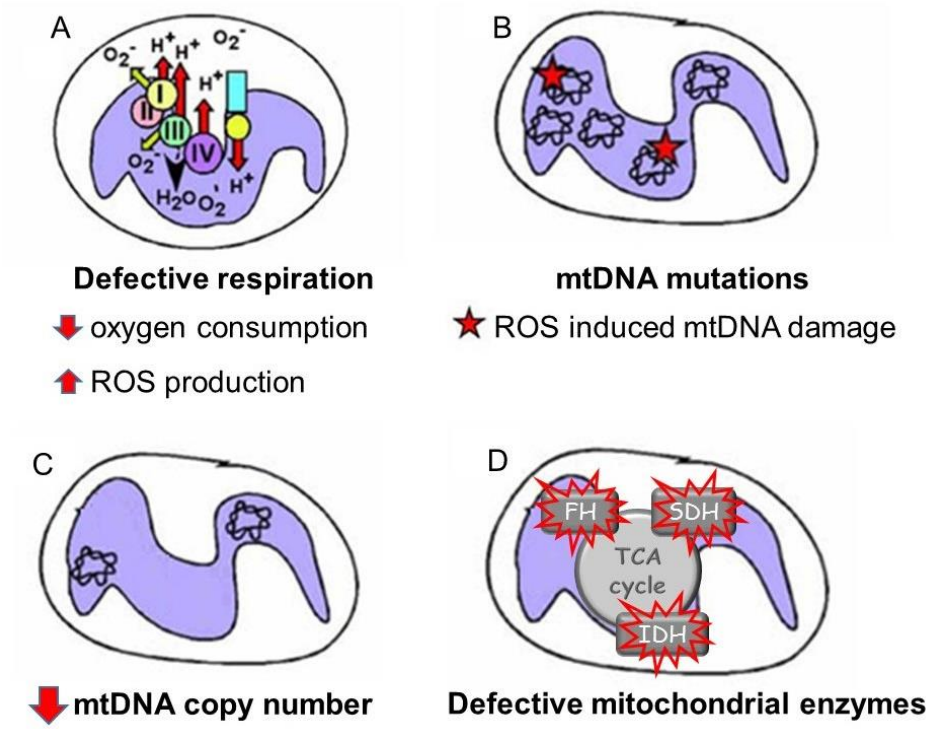


Figure 2.5.1 Types of Mitochondrial Dysfunction in Cancer Cells. Types of mitochondrial dysfunction include A) defective respiration which cause reduced oxygen consumption and increased ROS production; B) mtDNA mutations are frequently identified in cancer tissues and have a high potential to result in mitochondrial dysfunction and contribute to tumorigenesis; C) decreased mtDNA copy number, frequently detected in cancer tissues, is expected to affect energy production and increase ROS generation and cell survival, processes which are involved in cancer progression; and D) defects in the mitochondrial enzymes may result in deregulation of cellular energetics in cancer cells, which includes several enzymes of the TCA cycle such as Succinate dehydrogenase (SDH), Fumarate hydratase (FH), and Isocitrate dehydrogenase (IDH).[95]

of mitochondria in tumors plays an active role during disease development and progression.[96] Moreover, tumor cells and stem cells have a common ability to uncontrollably self-reproduce and possess an increased dependency on glycolysis for ATP production.[97] Proliferating tumor cells adapt to aerobic glycolysis whereas stem cells suppress their metabolism for maintaining their functionality.[97] Accordingly, glycolytic metabolism could be a therapeutic target through reversal of the abnormal metabolism of cancer cells by shifting it from glycolysis to glucose oxidation using dichloroacetate (DCA), a mitochondria-targeting small molecule. Indeed, DCA is reported to inhibit proliferation, induce apoptosis and suppress tumor growth by normalizing mitochondrial function.[98] Also, DCA was shown, for putative CD133+ glioblastoma stem cells, to prevent cancer initiating cells by inhibiting their mitochondrial activity.[99]

There are numerous molecules currently being tested in clinical trials that act on mitochondria. For instance, several clinically approved anticancer drugs such as paclitaxel, VP-16 (etoposide) and vinorelbine, as well as an increasing number of experimental anticancer drugs such as, ceramide, MKT077 and CD437, lonidamine, betulinic acid have been found to act directly on mitochondria to trigger apoptosis.[100] As these potential drugs usually do not exhibit adequate tumor and mitochondria-specific accumulation, an intensive effort to develop tumor-targeted mitochondria-specific approaches could be a significant help towards developing mitochondrial targets for cancer therapy. The selective accumulation approach to targeting tumor mitochondria requires two levels of specific accumulation; drug accumulation in the tumor and then drug accumulation in the mitochondria of cancer cells.[101] Conjugation of, one of the most commonly used

mitochondrotropic molecules, TPP cation has been used to deliver various bioactive cargos to mitochondria including antioxidants like coenzyme Q, ubiquinone, and various nitroxides, and also nucleic acids peptide and Cyclosporin A, which indicate the broad applicability of such an approach to mitochondria-specific delivery (Figure 2.5.2).[102] Also, TPP has been used in mitochondrial targeted photodynamic therapy to selectively target and inhibit Hsp90 activity in mitochondria of human cancer cells.[103] Overall, the possibility to have a ligand that can mediate both tumor-specific delivery and mitochondria-specific delivery inside the tumor cells is still in question. This could be attainable using a tumor-specific and mitochondria-specific nanocarrier to deliver an active molecule to mitochondria. The development of nanocarrier-based approaches becomes an integral part of mitochondria-targeted approaches to cancer therapy. Modified mitochondria-targeted nanocarriers are not only effective in the tumor-specific accumulation of a drug but also mediate mitochondria-specific accumulation within a tumor cell, which make them the ultimate tool in mitochondria-targeted anticancer approaches.

2.6 Potential Nano-therapy for Breast Cancer

2.6.1 Biological application and characterization of nanoparticles

The rapid developing field of nanotechnology including the biological application of nanoparticles have given rise to new diagnostic and therapeutic possibilities in the treatment of various cancer types. Recently, the use of conjugated nanoparticles allowed at least ten cancer-related proteins to be detected on tiny tumor sections, providing a new technique of analyzing the proteome of a distinct tumor, which potentially can increase the diagnosis and therapeutic efficacy.[104] Ultimately, the use of nanoparticles allows

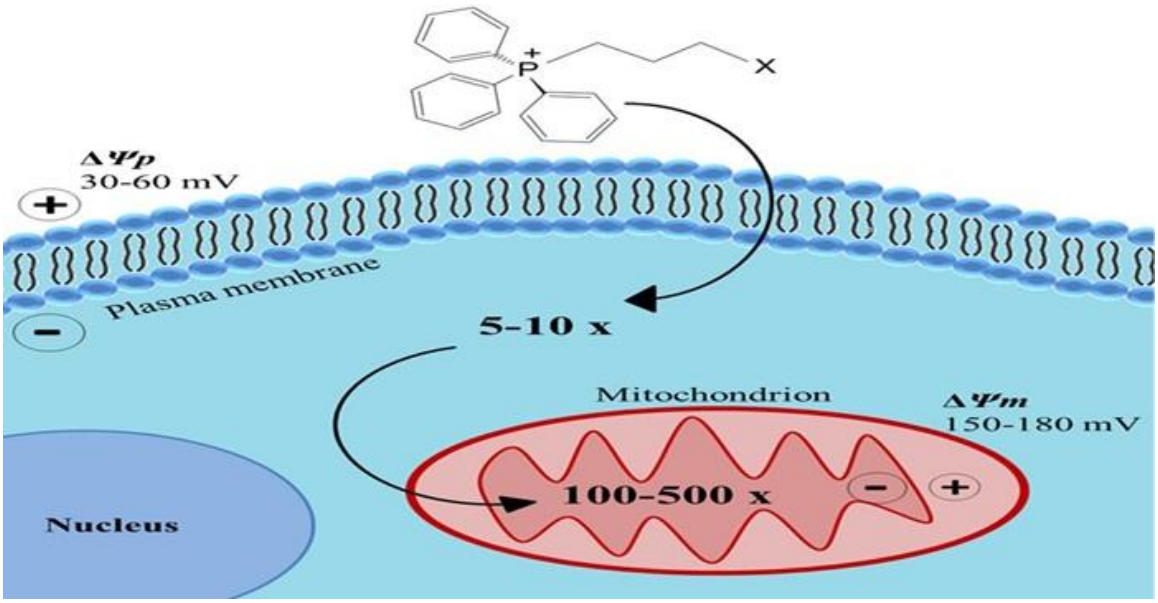


Figure 2.5.2 TPP Cation Accumulation in Mitochondria. The uptake of TPP cation into the cytoplasm (5-10 fold) is due to the negative plasma membrane potential (ΔΨp). Further accumulation of TPP from the cytoplasm into mitochondria (100-500 fold) is driven by the highly negative mitochondrial membrane potential (ΔΨm).

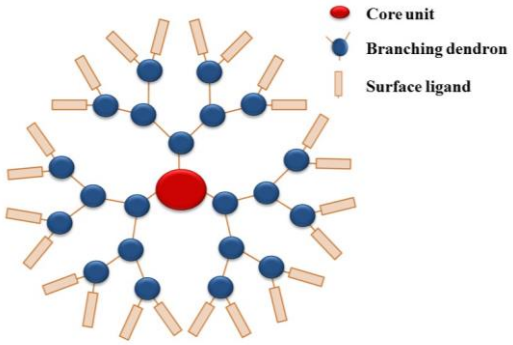
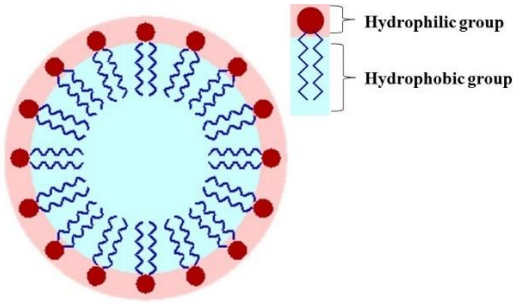
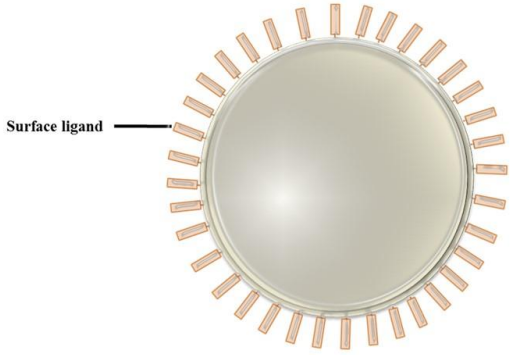
immediate tumor targeting and drug delivery in a unique fashion. Indeed, nanoparticles developed for drug delivery have many designs in terms of size, shape, and materials. Their characteristics usually differ in term of drug stability, loading capacity, release rate, and targeted delivery capability (Table 2.6.1.1).[104]

2.6.2 Targeting breast cancer using nanoparticles/nanocarriers

Nanomedicine focuses on application of nanotechnology in medicine for diagnosis, prevention, detection, and treatment of the disease. Targeted drug delivery system offers many potential benefits such as: (i) avoiding the side effects of the clinical formulation for improving solubility, (ii) protecting the entrapped therapeutic drug from degradation, (iii) modifying pharmacokinetic and tissue distribution profile to increase drug distribution in tumor, (iv) reducing toxicity to normal cells, and (v) increasing cellular uptake and internalization in cancer cells.[23] Moreover, besides use as drug delivery systems for cancer therapy, nanoparticles loaded with imaging agents were also found useful in imaging for tumor diagnosis.

2.6.2.1 Imaging and diagnosis of BC using nanoparticles/nanocarriers

Although magnetic resonance imaging (MRI) is considered very sensitive in detecting small tumors, its relative specificity tends to be low due to false positive signals.[105] The development of MRI contrast agents that are molecularly targeted may result in increased image specificity and clinical advantage. To this end there have been several studies that have targeted nanoparticles to breast cancer for enhanced breast cancer imaging.[105] Indeed, an interesting study has conjugated the amino terminal fragment of urokinase-type plasminogen activator (uPA) to magnetic iron oxide nanoparticles and

Nanoparticles	Shapes	Characteristics
Dendrimers	• Branched treelike. 	• Multifunctional central core. • Drug molecules attached to functional groups on the dendrimer surface.[106] • Host both hydrophobic and hydrophilic. • Useful delivery agents for genes and anticancer agents.[104]
Micelles	• Spherical or globular structures. 	• Form when constituent molecules with a hydrophobic end clump to form the central core. • Liquid environment. • Useful for delivery of water insoluble drugs carried in the hydrophobic central core.[106]
Nanospheres	• Spherical structures. 	• Composed of matrix system • Drug distributed by entrapment, attachment, or encapsulation. • Surface can be modified by the addition of polymers and biological materials. • Ligands or antibodies may be attached for targeting purposes.[107]

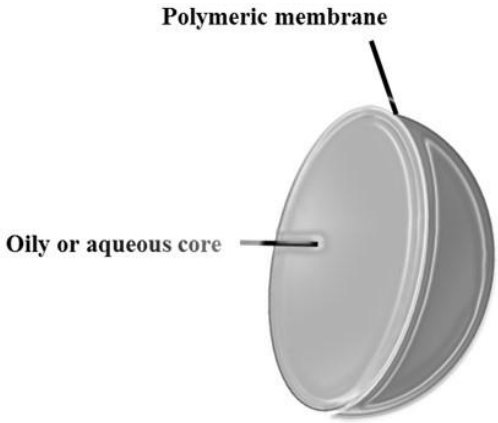
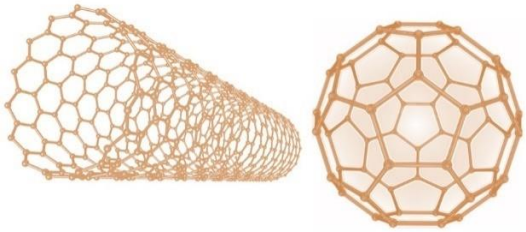
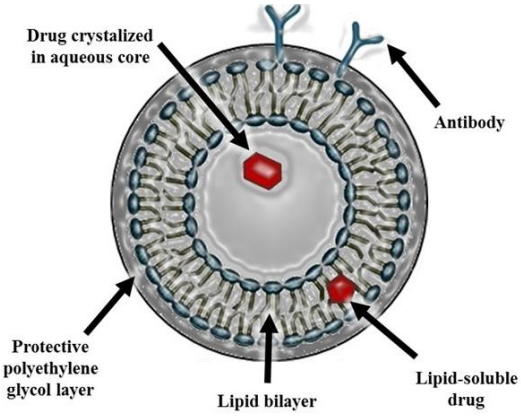
Nanocapsules	• Vesicular systems. 	• Central cavity or core to which drug confined. • Core is surrounded by an outer shell polymeric membrane to which surface bound targeting ligands or antibodies may be attached. • Core material may be solids, liquids, or gas. • Core environment may be aqueous or oily.[107]
Fullerenes & Nanotubes	• Hollow sphere or ellipsoid tube. 	• Composed of carbon atoms. • Atoms trapped inside fullerenes and tubes and antibodies or ligands bound to the surface for targeting.[108]
Liposomes	• Vesicles structure. 	• Composed of lipid layers.[104] Liposomes can disintegrate by electrostatic, hydrophobic, and van der Waals forces. • Coating particles with inert polymers (PEG) for steric stabilization.[109] • Surface coating allow liposomes to circulate for several days without clearance. • Liposomal vesicles release drug at the cell membrane and can access tumor cells at high concentrations. • This strategy reduces toxic side effects to normal tissue, while enhancing the therapeutic index of the delivered drug.[104]

Table 2.6.1.1. Nanoparticles Sizes, Shapes, and Materials.

demonstrated binding and internalization of these nanoparticles by uPA receptor expressing tumor cells.[110] Controlled delivery of the nanoparticles into mice bearing mammary tumors led to accumulation of particles in tumors and the generation of magnetic resonance contrast images that were detectable by a clinical MRI scanner.[110] Other strategies for enhanced MRI signals have included the use of folate receptor targeted magnetic oxide nanoparticles.[111]

Gold nanoshells (silica core surrounded by a thin gold shell) can be optically tuned with resonance that spans the visible and infrared spectrum.[112] By placing the peak absorption properties in the near infrared (NIR) region where tissue absorption is at minimum, nanoshells within tissue can preferentially absorb NIR light energy. Photothermal cancer therapy using nanoshell can be achieved by (i) the accumulation of nanoshells in the tumor and then (ii) the generation of heat from particle absorption of NIR light, which will eventually lead to tumor destruction. Utilizing a targeting strategy of conjugation of HER-2 antibody to NIR absorbing nanoshells, investigators have shown photothermal destruction of breast cancer cells.[113] In addition, in a mouse model, nanoshell treated tumors completely regressed after NIR illumination.[114] In an effort to further exploit the advantages of nanoparticles, investigators have demonstrated the *in vivo* ability to introduce multiple diagnostic capabilities in photothermal therapeutic nanocomplexes by simultaneously enhancing both NIR fluorescence and magnetic resonance imaging.[115] These multimodal nanoparticles have been conjugated to HER-2 antibodies and been shown to specifically, although not exclusively, accumulate in HER-2-positive breast tumors *in vivo*.[115]

2.6.2.2 Targeted drug delivery system

Lipid-based nanoparticles have attracted great attention as a drug delivery system due to their attractive biological properties such as good biocompatibility, biodegradability, low immunogenicity, and the ability to deliver hydrophilic and hydrophobic drugs. Indeed, liposomes are the most widely used and studied examples, with bilayer membrane structures composed of phospholipids for stabilizing drugs, directing their cargo toward specific sites, and for overcoming barriers to cellular uptake.[116] PEGylated lipids in the liposomes was developed to protect liposomes from destruction by the reticuloendothelial system (RES), thus to increase circulation time and increase drug accumulation in the tumors. Moreover, Doxil/caelyx, a PEGylated liposome formulation of the anticancer drug doxorubicin (DOX), was the first formulation approved for application in the clinic.[117] Targeted lipid-coated nanogels drug delivery platform can: (i) encapsulate a wide range of drug chemotherapeutics, (ii) display targeting ligands, and (iii) enhance drug retention within the nanogel core after photo-crosslinking and (iv) retain therapeutic activity after lyophilization allowing for long term storage.[118] Integrin αvβ3-targeted lipid-coated nanogels with cross-linked human serum albumin in the core were used for carrying various cargoes including paclitaxel, docetaxel, bortezomib, sorafenib and sunitinib. These particles exhibited potent activity in tumor cell viability assays with different drugs.[118] Although the work on modification of liposomes has achieved great progress, the application of liposomes in the clinic still poses several challenges including rapid clearance from the bloodstream, instability of the carrier, high production cost, and fast oxidation of some phospholipids.

Polymer-based nanoparticles show enormous potential for treating disease or repairing damaged tissues especially for cancer treatment, which relies on their remarkable properties including small size, excellent biocompatibility and biodegradability, prolonged circulation time in the bloodstream, enhanced drug loading capacity, and easy chemical modification or surface functionalization.[23] In general, polymer-based nanomedicine can be categorized into three groups based on drug-incorporation mechanisms including: polymer-drug conjugates by covalent conjugation, polymeric micelles by hydrophobic interactions, and polymersomes by encapsulation. Most of the polymers; such as poly(lactide), copolymer poly(lactide-co-glycolide), and PEG, are approved by the FDA as commonly explored carriers for targeted drug delivery.[23]

Protein-based nanomedicine has been given serious attention due to their biocompatibility, biodegradability as well as enhanced efficacy and low toxicity during treatment. Indeed, protein-based nanomedicine usually consists of natural protein subunits of the same protein or the combination of natural or synthetic proteins, and different types of drug molecules. There are a variety of proteins used drug delivery systems such as plant-derived viral capsids, small heat shock protein cages, albumin, soy and whey protein, casein, collagen and the ferritin/apoferritin protein cage.[119] Albumin may be used as a multipurpose protein carrier for improving drug targeting and pharmacokinetic properties.[120] As such, it is playing a vital role in the development of protein-based nanoparticles.[120] It demonstrates important features of stability in a broad range of pH (4-9) and temperature (4 °C - 60 °C), preferential uptake by the tumor, and is non-toxic.

Methotrexate-albumin conjugate, albumin-binding prodrug of DOX, and albumin PTX nanoparticle (Abraxane) have been designed and are currently in clinical trials.[120]

2.7 Conclusion

The BCSCs concept of self-renewal, pluripotency and tumorigenicity suggests the existence of small population of CSCs with increased tumor-initiating ability, tumor relapse, metastasis, and resistance to therapies. An interesting connection between CSCs and mitochondria prevails, as mitochondrial dysfunction apoptosis is reported to be associated with tumor resistance in conventional chemotherapy.[93,121] Considering the biology, molecular characteristics, and mechanisms of the tumor may help reaching a better treatment with increased efficacy and low toxicity. Therefore, therapy targeting mitochondria and specific CSCs signaling pathway may trigger cell death signaling cascades, mitochondria-dependent apoptosis, and reach total CSCs and tumor elimination.

The complex nature of CSCs features and mitochondrial functions, including signaling pathways and microenvironment, give rise to challenges in selecting the targeted element and whether individual or multiple targeting strategies should be used. In this regard, nanoparticle-based therapy, using nanoparticles/nanocarriers, seems to have the ability to combine various bioactive molecules, specifically, and safely reaching tumor foci with enhanced efficacy and low toxicity. The development of effective nanoparticle-based therapy for BCSC needs to be optimized through nanoparticles modeling combined with experimental validation for ultimate efficiency. The possibility of achieving this goal is still a hypothesis and further investigation is needed in this field.

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CHAPTER 3

INFLUENCE OF ESTROGEN ON MITOCHONDRIA IN ESTROGEN-DEPENDENT BREAST CANCER

3.1 Abstract

17 β-estradiol (E2) is a major risk factor for the development of breast cancer. Estrogen, through increasing mitochondria-derived reactive oxygen species (ROS), contributes to estrogen-induced breast carcinogenesis. However, the exact mitochondrial mechanisms underlying the estrogen carcinogenic effect in breast tissue are unknown. The aim of this study was to elucidate the mechanisms by which mitochondria contribute to the growth of breast cancer. Triphenylphosphonium (TPP), a lipophilic cation whose selective accumulation in the mitochondria is driven by both plasma and mitochondrial membrane potentials, was used in this study to check whether an imbalance in mitochondrial bioenergetics, in part, may be responsible for estrogen-induced growth of breast cancer. TPP has been shown to rapidly accumulate and be retained by MCF-7 cells, and inhibit oxidative phosphorylation in mitochondria. In principle, factors other than membrane potential may also play a role to counteract estrogen effects on the growth of breast cancer cells. Our results showed that TPP inhibited E2-induced increase of cell viability, growth, proliferation, ROS formation, and mitochondrial membrane potential of serum deprived estrogen-dependent MCF-7 breast cancer cells. Our results also demonstrated that TPP decreased E2-induced DNA damage and mitochondrial ROS production in MCF-7 cells. Although demonstrated in a breast cancer cell line, these results indicate that E2 modifies mitochondrial dynamics, biogenesis and metabolism, and thus compromises the normal development and function of mitochondria in cancer tissues. Furthermore, our findings offer a new perspective on the utility of mitochondria-targeted lipophilic cations, such as

TPP, as a promising new class of targeted-drugs for the treatment of estrogen-dependent breast tumors.

3.2 Introduction

Estrogens play a major role in the initiation and progression of breast cancer. A large body of evidence, both epidemiological and clinical, has linked elevated lifetime exposure to estrogens with increased risk of developing breast cancer.[1–3] However, the exact mechanisms underlying the carcinogenic effect of estrogen in breast tissue are not clearly understood. Estrogen receptor (ER)-mediated signaling pathways are considered as the most widely acknowledged mechanism of estrogen carcinogenicity.[4,5] Whilst, nearly 70% of newly diagnosed breast cancer patients are ER+, almost half of those patients do not respond to hormonal therapies.[6] This suggests that the growth of estrogen-dependent cells is regulated not only by ER-mediated signaling pathways, but also by ER-independent pathways.[7]

Mitochondria play a central role in cellular metabolism, calcium homeostasis, redox signaling, apoptosis and cell death.[8] Over the past decade, there has been extensive investigation into the role of mitochondria in regulating cell growth and proliferation.[8] Tumor cells are characterized by dysfunctional mitochondria, which is demonstrated by the transition of the source of metabolic energy from oxidative phosphorylation to active glycolysis and increased ROS production.[9] Estrogens, through increasing mitochondria-derived ROS, contribute to breast carcinogenesis, although the exact role of mitochondria

and ROS in the carcinogenic effect of estrogen in breast tissue is still extensively unexplored.[10] For instance, 17 β-estradiol (E2), the most potent endogenous estrogen, induces DNA synthesis in MCF-7 breast cancer cells through mitochondrial oxidant signaling.[10] Indeed, E2-induced mitochondrial ROS regulates cell cycle G1 to S transition via a nongenomic, ER-independent signaling pathway.[10] Therefore, targeting mitochondria as a cancer therapeutic strategy has attracted attention in recent years and multiple investigations and studies in this area have been conducted.[9] For instance, several studies have targeted mitochondria and mitochondria-associated molecules and pathways, including mitochondrial membrane potential, electron transport chain, and apoptotic pathway, as a potential cancer therapeutic targets.[9]

To achieve optimal targeting of therapeutic agents, tumor- and mitochondria-specific accumulation is necessary.[11] The Triphenylphosphonium (TPP) cation has been used to deliver various bioactive molecules to mitochondria including antioxidants, which indicates the broad applicability of such an approach to mitochondria-specific delivery.[12] TPP is a lipophilic cation that has the property of being lipid-soluble, even with its net positive charge. This property allows TPP to pass through the phospholipid bilayers of the cell membrane and mitochondria.[13] TPP conjugated to a therapeutic molecule will accumulate in the cytoplasm, about 5 to 10 fold compared to the extracellular environment, due to the driving force of the plasma membrane potential ($\Delta\Psi_p$, -30 to -60 mV).[13] Further accumulation of TPP conjugates, about 100 to 500 fold compared to the extracellular environment, is driven by the mitochondrial membrane potential ($\Delta\Psi_m$, approximately -180 mV).[13] However, TPP has been shown to produce an uncoupling effect.[14] Indeed,

cellular toxicity linked to TPP cation may limit its therapeutic potential.[15] For instance, TPP accumulation in the mitochondria is capable of disrupting its membrane integrity and altering the metabolic activities such as ATP synthesis and respiratory chain functions.[16] Moreover, TPP cation has shown to potentially affect mitochondrial bioenergetics by increasing proton leak, decreasing mitochondrial membrane potential and inhibiting respiratory chain complexes.[17]

In order to elucidate the mitochondria-targeted effect of E2 in breast cancer, we examined their inhibition by using a mitochondria-targeted agent TPP. We evaluated the cell viability, growth, proliferation, mitochondrial membrane potential, cellular ROS formation, DNA damage, and mitochondrial ROS production of estrogen-dependent breast cancer (MCF-7), metastatic breast cancer (MDA-MB231), and normal breast epithelial (MCF-10A) cells treated with E2, TPP, and co-treatment of E2 with TPP, *in vitro*.

3.3 Materials and methods

3.3.1 Chemicals and reagents.

17β-Estradiol (E2), (4-carboxybutyl) triphenylphosphonium bromide (TPP), dimethylsulfoxide (DMSO), 3(4,5-dimethylthiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT), sulforhodamine B (SRB), trichloroacetic acid (TCA), and H_2O_2 were all purchased from Sigma-Aldrich (St. Louis, MO, USA). A cell proliferation ELISA (BrdU-colorimetric) kit was obtained from Roche Diagnostics (Indianapolis, IN, USA). MitoSOX Red, MitoTracker Green, and propidium iodide (PI) were purchased from Thermo Fisher

Scientific (Waltham, MA, USA), I 2′,7′-Dichlorofluorescin diacetate (DCFDA) and all tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA), unless otherwise specified.

3.3.2 Cell culture.

The estrogen-dependent breast cancer (MCF-7), metastatic breast cancer (MDA-MB231), and normal breast epithelial (MCF-10A) cells were routinely cultured in phenol red DMEM-F12 media (1:1) supplemented with 5% (v/v) fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin (complete media) in a humidified atmosphere containing 5% CO_2 at 37 °C. The cell culture media, serum, and antibiotics were purchased from Invitrogen Corp, CA, USA. Culture media were changed to starvation media (serum free media + antibiotics) and cells incubated for 48 h prior to commencement of all experiments, unless otherwise indicated.

3.3.3 Cell viability.

MTT was used to determine cell viability according to the manufacturer's protocol.[18] In brief, MCF-7, MCF-10A, and MDA-MB231 cells were seeded in three separate 96-well plates (approx. 7500 cells/well) overnight in complete media and serum starved for 48 h prior to treatment with E2, TPP, and co-treatment of TPP with E2 for 48 h. Then, 20 µL of (5 mg/mL) MTT was added to each well. After incubation for 3.5 h at 37 °C, the media was carefully removed and 100 µL of MTT solvent (100% DMSO) was added to each well. The plates were then covered with aluminium foil and agitated on shaker for 15 min.

Absorbance was recorded at 590 nm (reference λ at 620 nm) on a Tecan Genios microplate reader.

3.3.4 Cell growth.

SRB was used to determine cell growth as described by Skehan et al.[19] Briefly, cells were seeded in three separate 96-well plates (approx. 7500 cells/well) for 24 h in complete media and serum starved for 48 h prior to adding the various cell treatments for 48 h. The cells were then fixed with 50% TCA solution, layered on top of the existing medium, and chilled to 4 °C. After 1 h of incubation at 4 °C, the plates were rinsed four times with distilled water. Then 100 µL of 0.4% (w/v) SRB was added to cover the bottom of the well and allowed to stain for 20 min. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl) aminomethane]. Absorbance was determined at 590 nm (reference λ at 620 nm) on a Tecan Genios microplate reader.

3.3.5 Cell proliferation.

BrdU incorporation assay was used to assess cell proliferation. A colorimetric BrdU cell proliferation assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). The BrdU Cell Proliferation Assay Kit detects BrdU incorporated into cellular DNA during cell proliferation using an anti-BrdU antibody, which is an indicator of the newly synthesized DNA in proliferating cells or repair of damaged cells.[20] Briefly, MCF-7 cells were grown in 96-well plates until 50% confluent in complete media,

and serum starved for 48 h followed by the various cell treatments for 48 h. Absorbance was measured by a Tecan Genios microplate reader at 450 nm (reference λ at 700 nm).

3.3.6 Mitochondrial bioenergetics.

The rhodamine (Rh123)-mitochondrial membrane potential assay was used to evaluate mitochondrial bioenergetics. The Rh123 fluorescent dye distributes according to the negative membrane potential across the mitochondrial inner membrane.[21] Loss of potential will result in loss of the dye and therefore fluorescence.[21] In brief, cells were seeded into black sided 96-well flat bottom plates (Thermo Fisher Scientific Inc, MA, USA) overnight in complete media and serum starved for 48 h prior to adding the various cell treatments for 48 h. Followed by an addition of 10 µL of 0.1 µg/mL of Rh123 to each well. The cell suspension was gently agitated and incubated at 37 °C for 30 min in the dark. The fluorescence of Rh123 was measured on a Tecan Genios microplate reader using 503 nm and 527 nm as excitation and emission filters, respectively.

3.3.7 Cellular ROS production.

DCFDA was used to determine cellular ROS production as described by Felty et al.[22] Briefly, cells were seeded into black sided 96-well flat bottom plates and allowed to adhere overnight. Post seeding, cells were serum starved for 48 h after which they were treated for 48 h followed by incubation with 10 µM DCFDA for 20 min. The oxidized products were measured on a Tecan Genios microplate reader using 485 nm and 535 nm as excitation and emission filters respectively.

3.3.8 Mitochondrial ROS detection.

MitoSOX Red mitochondrial superoxide indicator was used to estimate the amount of superoxide anion produced in mitochondria. The analysis was performed according to the method described by Hahm et al.[23] with the use of confocal microscopy and fluorescence microplate reader. Briefly, cells were seeded into 96-well plate and allowed to adhere for an overnight. Post seeding, cells were serum starved for 48 h after which they were treated for 48 h followed by incubation with 5 µM MitoSOX Red for 30 min. Cells were washed with phosphate-buffered saline (PBS) and fluorescence was detected using a Gemini EM microplate spectrofluorometer (Ex/Em: 510/580 nm). For immunofluorescence staining, cells were seeded and treated in chamber slides. After treatment, cells were incubated with 5 µM MitoSOX Red for 30 min at 37°C. Cells were then incubated with 200 nM MitoTracker Green for 15 min, to stain mitochondria. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT) and examined under a Nikon C1 laser scanning confocal microscope at 40× objective magnification.

3.3.9 DNA damage assessment.

Single cell gel electrophoresis (the comet assay) was used to measure DNA damage as described by Karbaschi & Cooke.[24] Briefly, cells were seeded into 6-well plates and allowed to adhere overnight. Post seeding, cells were treated for 3 h or 24 h, on RT, and then processed for alkaline comet assay. In brief, cells were immersed in 0.6% low melting point agarose, embedded on pre-coated slides with 1% normal melting point agarose, and lysed overnight at 4 °C. The slides were washed and then incubated with alkaline electrophoresis solution for 20 min. Subsequently, electrophoresis was carried on in the

same solution for 20 min at 25 V. After electrophoresis, the slides were incubated with neutralization buffer (0.4 M Tris-base, pH 7.5) for 20 min, washed and subsequently dried and stained with propidium iodide (PI, 2.5 μg/mL) for 20 min. The images of comets were visualized by using an on-line CCD camera, fluorescence microscopy at 40× magnification, and Comet Assay IV software (Perceptive Instruments, Suffolk, UK). 100 cells were scored for each treatment and the percentage of tail DNA was calculated for each nucleoid image.

3.3.10 Statistical Analysis.

Statistical analyses was performed using GraphPad Prism, version 6 (GraphPad, CA, USA). The data was evaluated using one-way ANOVA and Tukey's multiple comparisons tests to compare the values between groups. The level of statistical significance was set at $p < 0.05$.

3.4 Results

E2-induced cellular viability is inhibited by TPP in estrogen-dependent breast cancer cells.

We initially tested the effect of E2, TPP, and co-treatment of E2 with TPP on the viability of MCF-7, MDA-MB231, and MCF-10A cells in serum-free conditions. Cellular viability was assessed by MTT assay, which determines mitochondrial activity.[25] As shown in Figure 3.4.1, a significant increase in cell viability was observed for both E2 100 pg/mL ($p < 0.0001$) and E2 100 ng/mL ($p < 0.0001$), compared to control (0.1% DMSO), for MCF-7

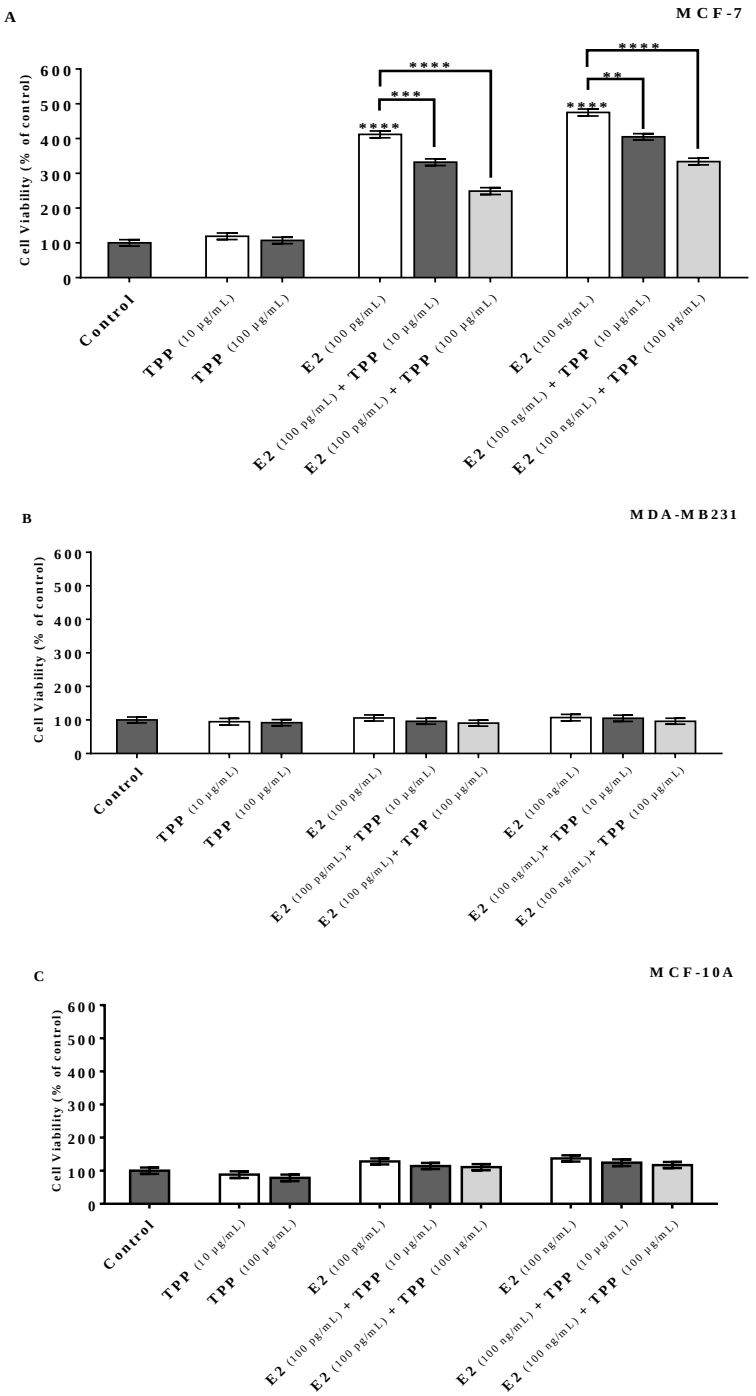


Figure 3.4.1 E2 increases viability and co-treatment with TPP decreases E2-induced viability in MCF-7 cells, determined by the MTT assay. Cell viability for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatment with E2, TPP, or co-treatment of TPP with E2 for 48 h. Results are expressed as mean ± S.E.M. of three independent experiments expressed relative to the control (0.1% DMSO) set as 100% cell viability. ***($p < 0.001$) and ****($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

cells (Figure 3.4.1A). This increase was not observed for MDA-MB231 (Figure 3.4.1B) nor MCF-10A (Figure 3.4.1C) cells. However, neither concentration of TPP (10 µg/mL or 100 µg/mL) influenced cell viability of MCF-7, MDA-MB213, and MCF-10A cells. Interestingly, the results also showed that co-treatment of E2 with TPP had a significant decrease in the cell viability of MCF-7 cells. Treatment with 100 pg/mL E2 was significantly inhibited, (p <0.001), by co-treatment with 10 µg/mL TPP. This effect was more pronounced at the higher concentration of TPP (100 µg/mL, p <0.0001). A higher concentration of E2, 100 ng/mL, was also inhibited by co-treatment with 10 µg/mL TPP (p <0.01), and a more significant inhibition by co-treatment with 100 µg/mL TPP (p <0.0001). The positive control (5% FBS media) showed a significant increase in the cell viability for all cell lines (p <0.0001); however, no significant effect was observed in serum free media compared to control (Figure 3.4.1.S). The serum-free conditions used in these experiments were optimal for observing E2-induced effect on breast cancer cells, since serum deprivation synchronizes cells in the G0/G1 phase of the cell cycle.[22] These findings show that E2 induces cellular viability and that inhibited by treatment with TPP.

E2-induced cell growth is inhibited by TPP in estrogen-dependent breast cancer cells.

To confirm the inhibition of MCF-7 cell viability by co-treatment of E2 with TPP, SRB assay was used. This detects cellular protein, to determine the cell growth for the three cell lines. A significant increase in cell growth was noted for both E2 concentrations (p <0.0001) on MCF-7 cell (Figure 3.4.2A). However, this was not noticed for MDA-MB231 (Figure 3.4.2B) nor MCF-10A (Figure 3.4.2C) cells. Nevertheless, there was no observable cell growth effect with either TPP concentrations on the three cell lines (Figure 3.4.2).

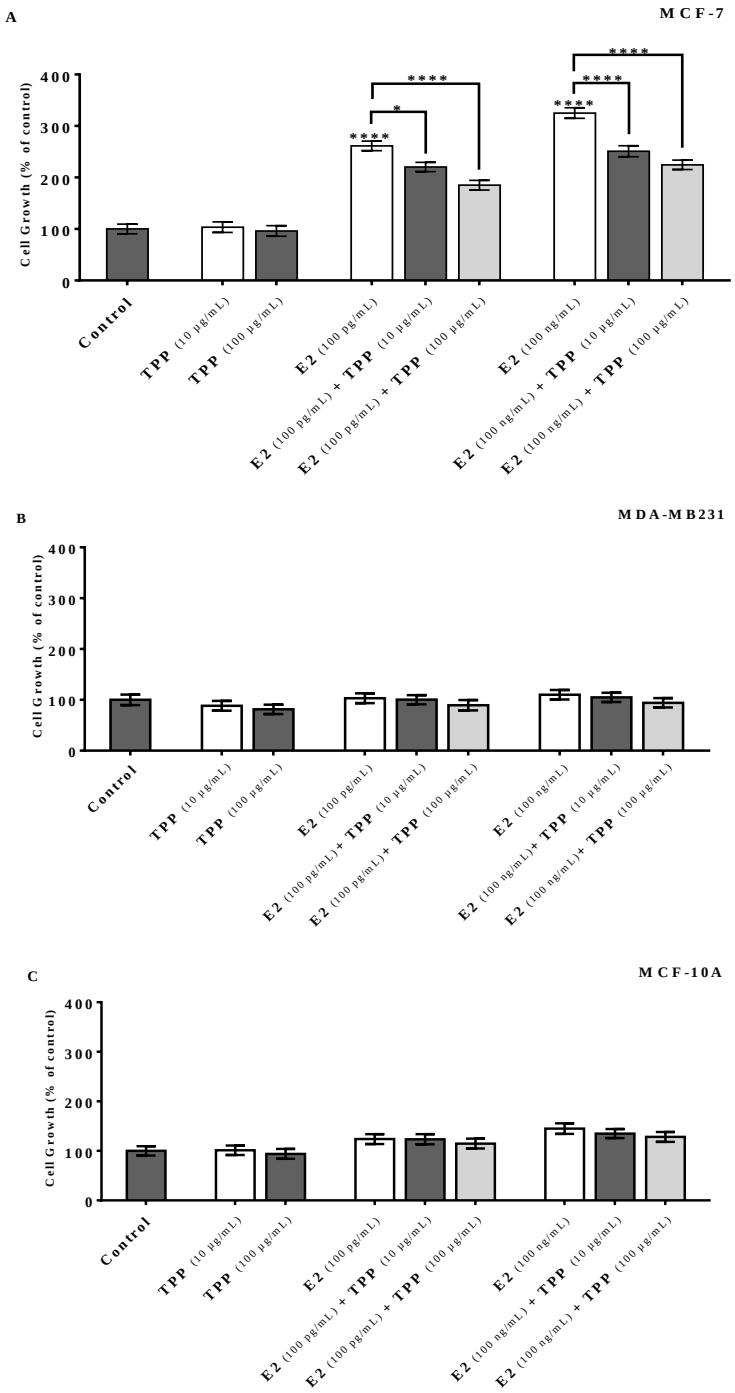


Figure 3.4.2 E2 increases growth and co-treatment with TPP decreases E2-induced growth in MCF-7 cells, determined by SRB assay. Cell growth for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatment with E2, TPP, or co-treatment of TPP with E2 for 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments expressed relative to the control (0.1% DMSO) set as 100% cell growth. *($p < 0.05$) and ****($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

Intriguingly, when E2 was co-treated with TPP, a significant inhibition in cell growth was observed for MCF-7 cells. The treatment with 100 pg/mL E2 was inhibited by co-treatment with 10 µg/mL TPP (p <0.05), and further significant inhibition by co-treatment with 100 µg/mL TPP (p <0.0001). Additionally, the higher concentration of E2, 100 ng/mL, was significantly inhibited by co-treatment with 10 µg/mL and 100 µg/mL TPP (p <0.0001). 5% FBS media significantly increased cell growth (p <0.0001), whereas, serum free media did not show any effects on cell growth on all cell lines (Figure 3.4.2.S). These results demonstrate that E2 induces cell growth and that inhibited by treatment with TPP.

E2-induced proliferation is decreased by TPP in estrogen-dependent breast cancer cells.

To further confirm the inhibition of MCF-7 cell viability and growth by co-treatment of E2 with TPP, we tested whether the co-treatment induces a similar effect on cell proliferation and DNA synthesis using the BrdU cell proliferation assay, which detects BrdU incorporation into cellular DNA during cell proliferation.[20] As shown in Figure 3.4.3, both E2 concentrations significantly stimulated the proliferation of MCF-7 cells (p <0.0001). However, the treatments of both TPP concentration had no effect on the proliferation of MCF-7 cells. Interestingly, both co-treatments of E2 with 100 µg/mL TPP significantly inhibited E2-induced DNA synthesis and cell proliferation (p <0.05) compared to E2 concentrations. As expected, 5% FBS media increased cell proliferation and DNA synthesis (p <0.0001), while, serum free media did not show any effects on cell proliferation for MCF-7 cells. Therefore, our findings show that E2 promotes DNA synthesis and cell proliferation and this inhibited by treatment with TPP.

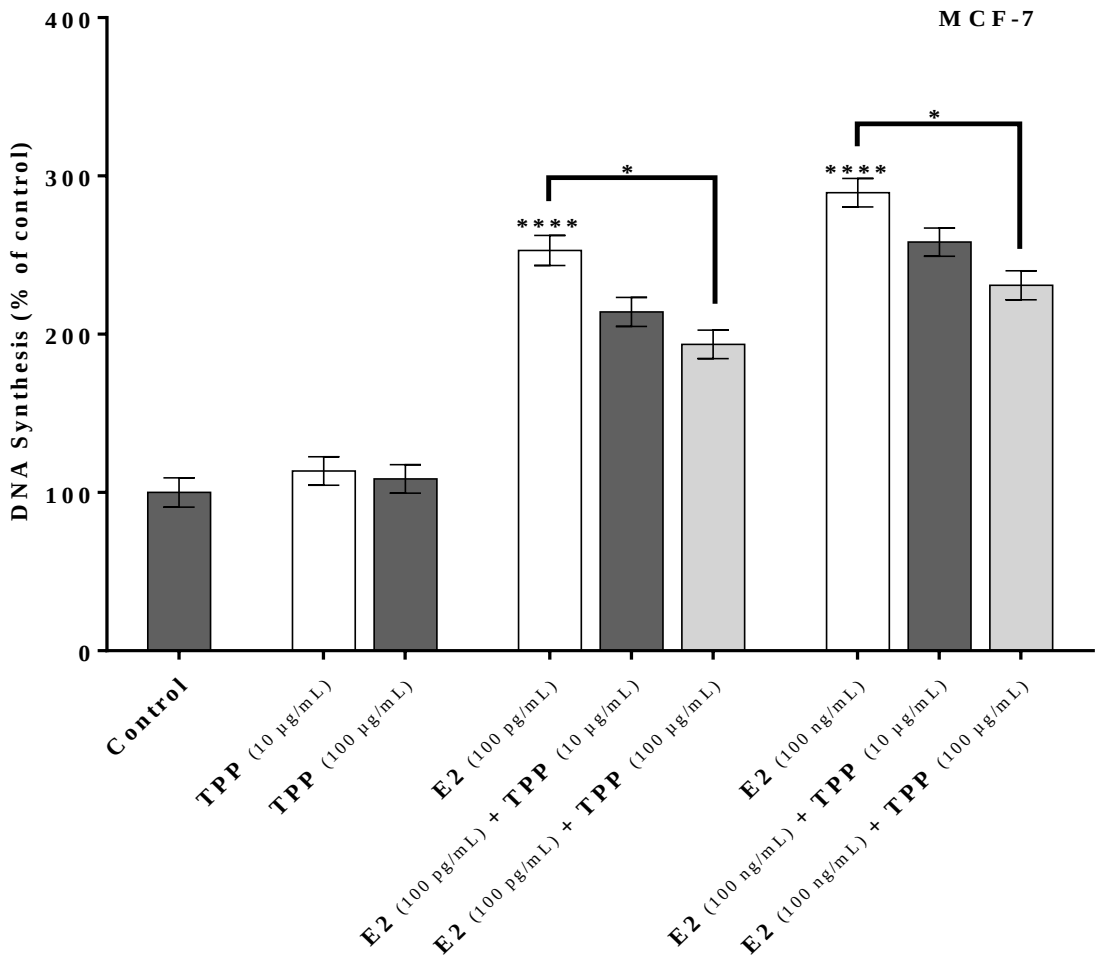


Figure 3.4.3 E2 increases DNA synthesis and co-treatment with TPP decreases E2-induced DNA synthesis in MCF-7 cells, determined by BrdU cell proliferation assay. MCF-7 cells were seeded and grown in 96-well plates until 50% confluent in complete media then serum starved for 48 h followed by the various treatments for 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments expressed relative to the control (0.1% DMSO) set as 100% DNA synthesis. **($p < 0.01$) and ****($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

TPP treatment induces changes in E2-induced mitochondrial bioenergetics in estrogen-dependent breast cancer cells.

Uptake of the lipophilic cation, TPP, has been often used as a membrane potential probe, because it is considered to produce an uncoupling effect.[14] The possibility that TPP might alter E2-induced mitochondrial bioenergetics, via their action at $\Delta\Psi_m$[17], was assessed, using Rh123-mitochondrial membrane potential assay. As shown in Figure 3.4.4, both E2 concentrations had significantly increased the Rh123-positive mitochondrial membrane potential (p <0.0001) for MCF-7 cells (Figure 3.4.4A). However, this effect was not observed in MDA-MB231 (Figure 3.4.4B) or MCF-10A (Figure 3.4.4C) cells. Furthermore, treatments with both TPP concentrations did not alter the mitochondrial membrane potential for all cell lines. Remarkably, co-treatment of E2 with TPP induces loss of $\Delta\Psi_m$ of MCF-7 cells. The treatment with 100 pg/mL E2 was significantly reduced by the co-treatment with 100 µg/mL TPP (p <0.001), but not with 10 µg/mL TPP. Also, the 100 ng/mL E2 was significantly decreased by the co-treatment with 10 µg/mL TPP (p <0.01) and 100 µg/mL TPP (p <0.0001), in a dose-dependent manner (Figure 3.4.4A). Thus, these findings show that E2 induces an increase in mitochondrial membrane potential and this decreased by treatment with TPP in MCF-7 cells.

E2-induced ROS production is decreased by TPP in estrogen-dependent breast cancer cells.

Since mitochondria are considered a major source of ROS, and treatment with TPP decreased E2-induced mitochondrial bioenergetics, cellular ROS production was investigated using DCFDA. This assay detects the generation of ROS including:

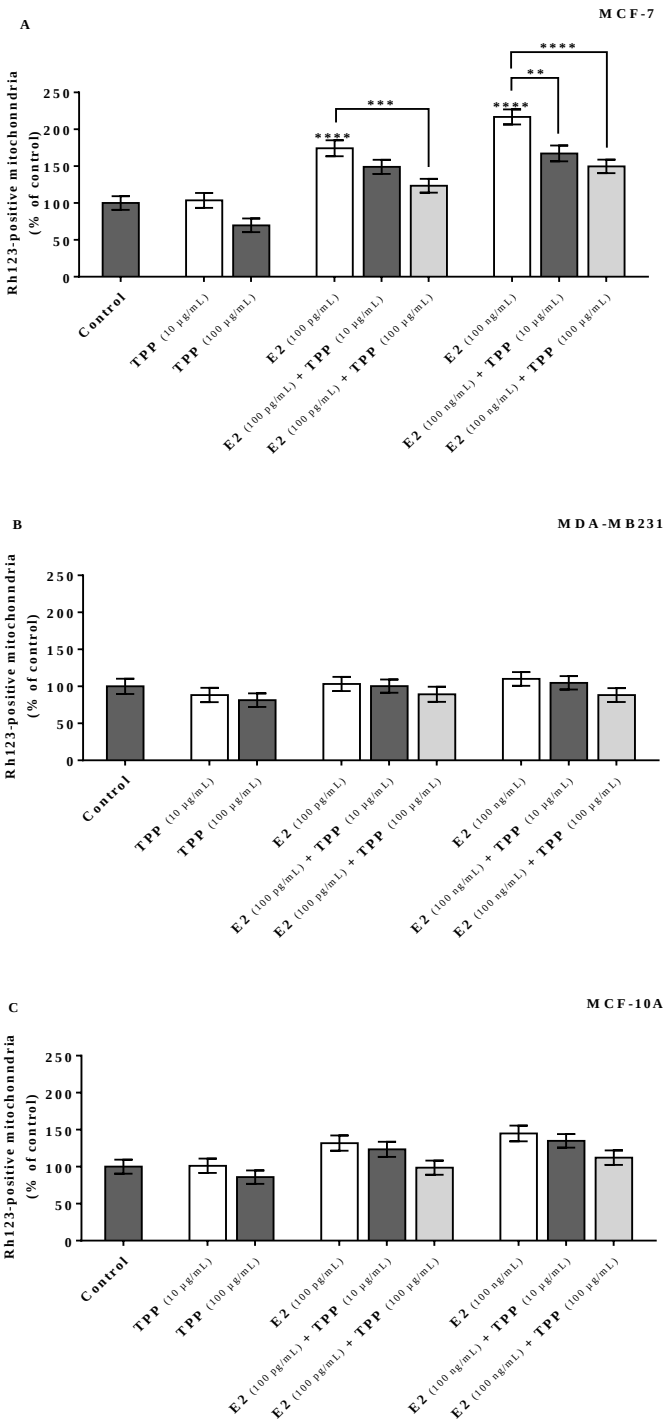


Figure 3.4.4 E2 increases mitochondrial membrane potential and co-treatment with TPP decreases E2-induced mitochondrial membrane potential in MCF-7 cells, determined by mitochondrial membrane potential assay. Rh123-positive $\Delta\Psi_m$ for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to the various cell treatments for 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments expressed relative to the control (0.1% DMSO) set as 100% Rh123-positive mitochondria. **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

superoxide; hydrogen peroxide; hydroxyl radical and other ROS within the cell.[26] As shown in Figure 3.4.5, both E2 concentrations, 100 pg/mL and 100 ng/mL, significantly increased ROS generation in MCF-7 cells (Figure 3.4.5A). However, both concentrations of TPP did not show an effect on ROS production of MCF-7 (Figure 3.4.5A), MDA-MB213 (Figure 3.4.5B), or MCF-10A (Figure 3.4.5C) cells. Remarkably, co-treatment with TPP decreased E2-induced ROS production in MCF-7. Only the higher concentration of TPP, 100 µg/mL, was able to significantly decrease the E2-induced ROS production of both E2 treatments, 100 pg/mL (p <0.01) and 100 ng/mL (p <0.05), in MCF-7 cells (Figure 3.4.5A). Thus, these results show that E2 increases ROS production and this could be decreased by treatment with TPP.

E2-induced mitochondrial ROS production is decreased by TPP in estrogen-dependent breast cancer cells.

Since the treatment with TPP decreased E2-induced cellular ROS, further investigation was needed to localize the source of ROS production. Mitochondrial ROS production was detected using MitoSOX Red mitochondrial superoxide indicator, which detects the superoxide anion produced in mitochondria. As shown in Figure 3.4.6A, E2 treatment caused an increase (p <0.0001) in MitoSOX Red fluorescence in MCF-7 cells compared to control (0.1% DMSO). However, TPP did not show an effect on mitochondrial ROS production in MCF-7 cells. Astonishingly, co-treatment with TPP decreased E2-induced MitoSOX Red fluorescence (p <0.0001). The positive control rotenone, an inhibitor of the mitochondrial complex I, showed a significant increase in MitoSOX Red fluorescence (p < 0.0001) (Figure 3.4.6A). ROS generation by E2 and TPP treatments in MCF-7 cells was

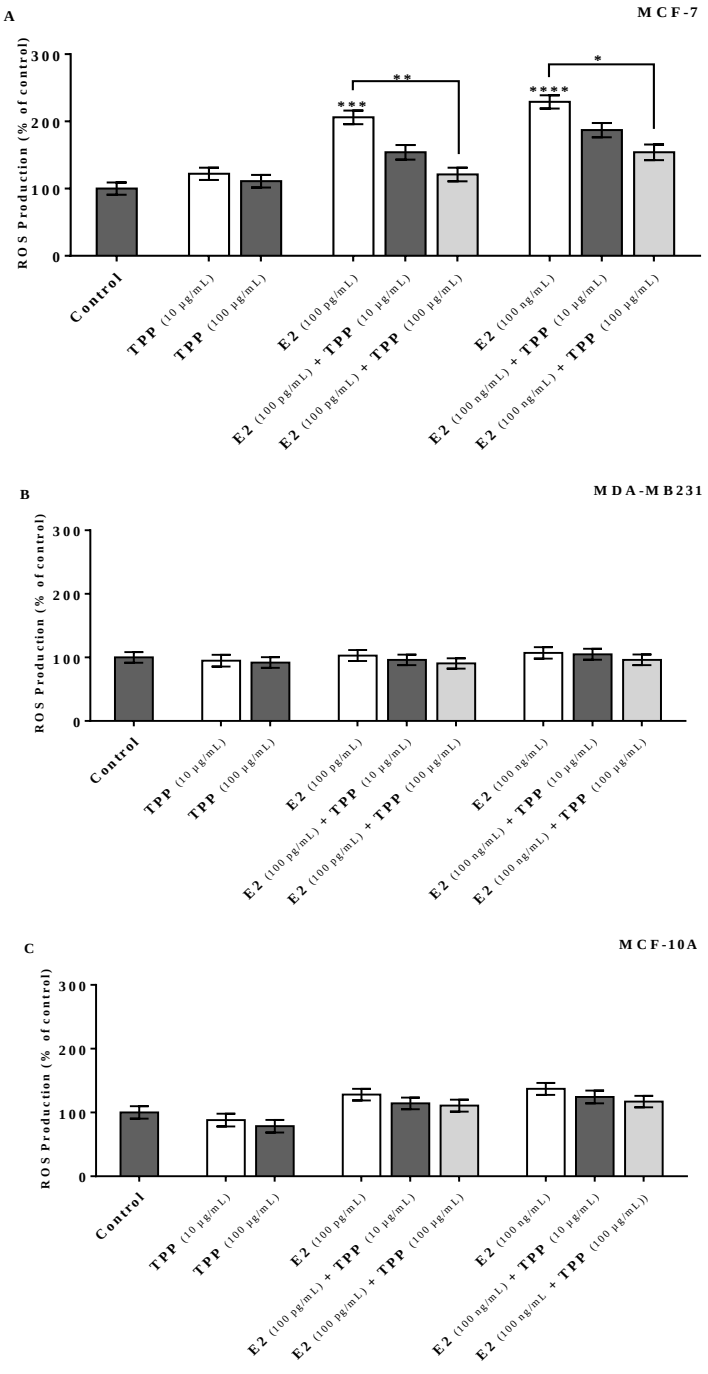
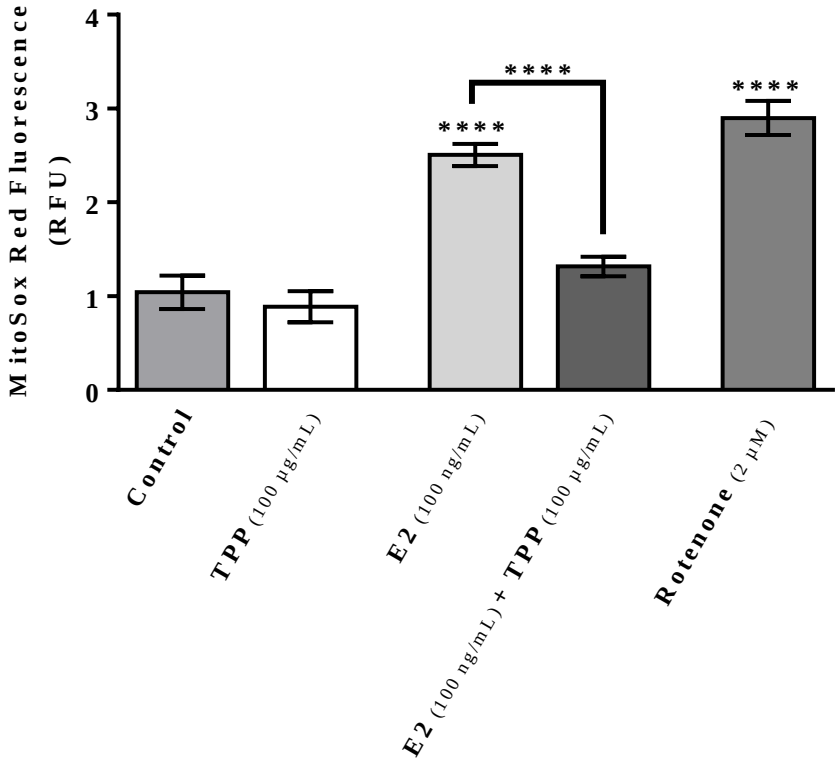


Figure 3.4.5 E2 increases ROS production and co-treatment with TPP decreases E2-induced ROS production in MCF-7 cells, determined by DCFDA ROS formation assay. ROS production for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatments for 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments expressed relative to the control (0.1% DMSO) set as 100% ROS production. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

A.


MitoSox Red Fluorescence (RFU)
0
1
2
3
4
****
****
****
Control
TPP (100 µg/mL)
E2 (100 ng/mL)
E2 (100 ng/mL) + TPP (100 µg/mL)
Rotenone (2 µM)

B.

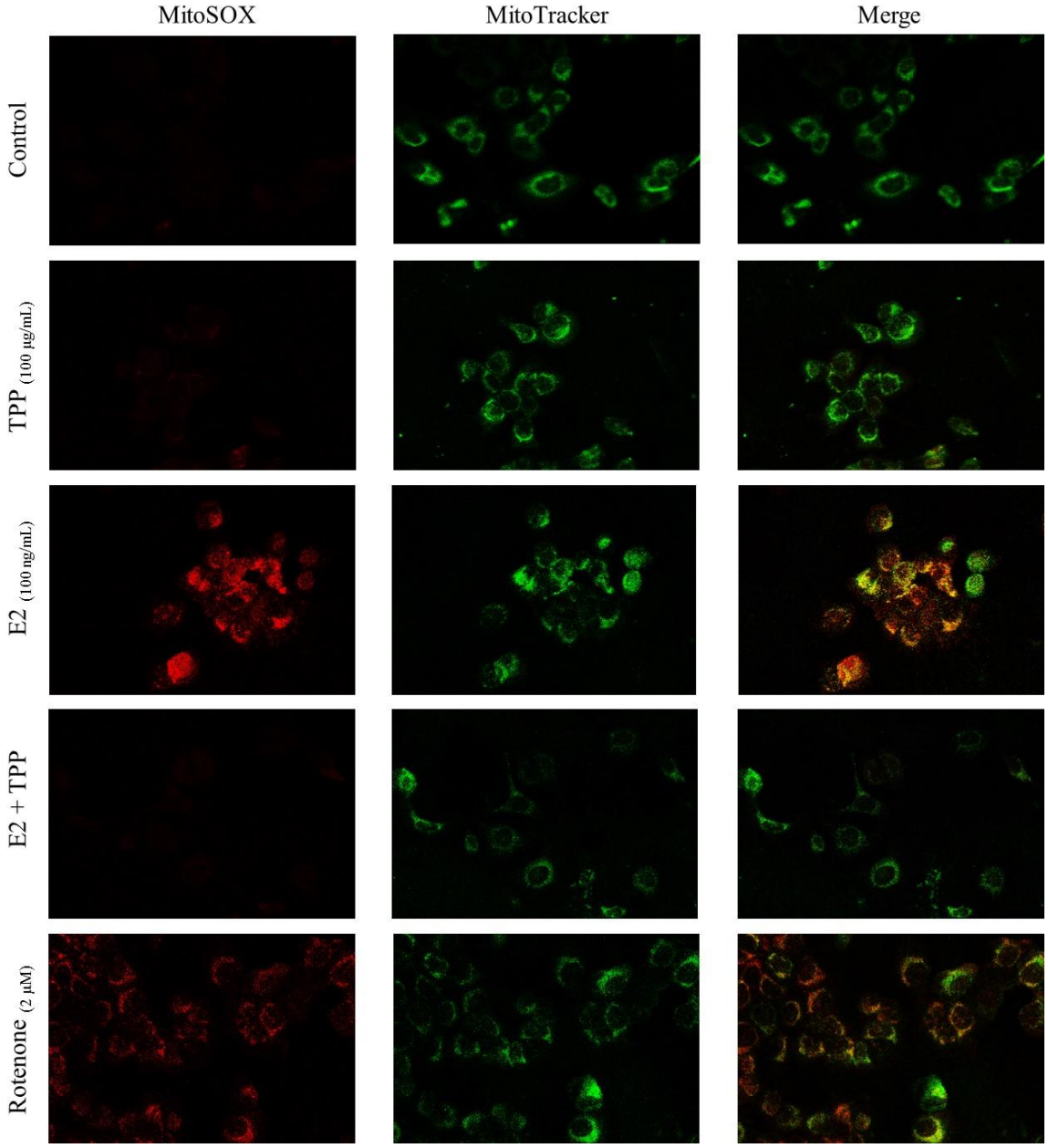
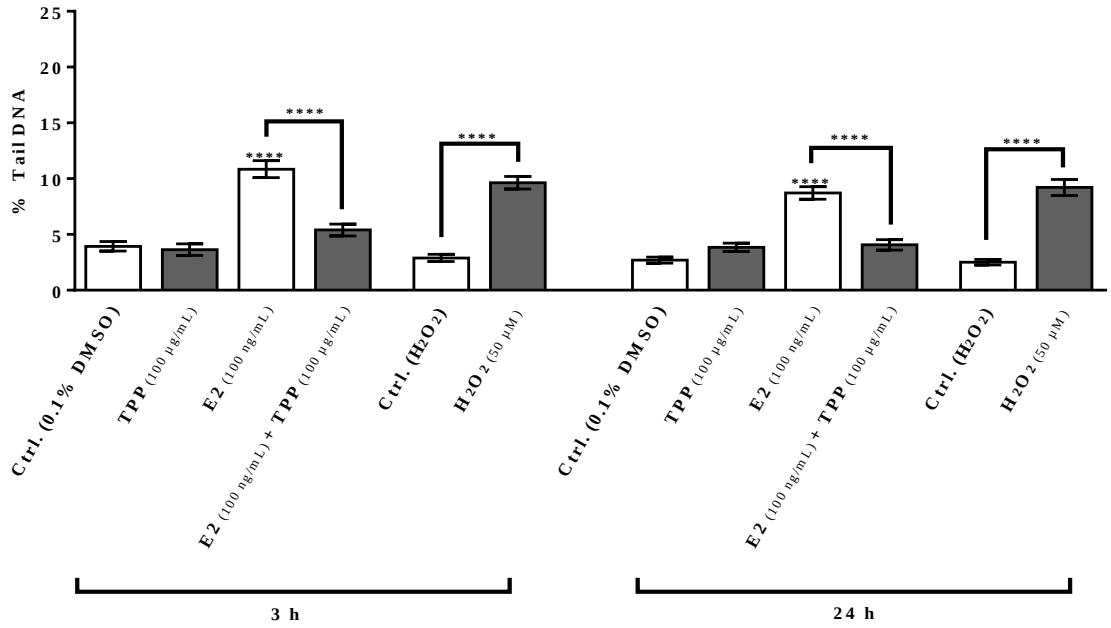


Figure 3.4.6 E2 increases mitochondrial ROS production and co-treatment with TPP decreases E2-induced mitochondrial ROS production in MCF-7 cells, determined by MitoSOX Red Mitochondrial Superoxide Indicator assay. Cells were cultured for 24 h in complete media, serum starved for 48 h prior to treatments for 48 h, and then processed for MitoSOX Red assay. (A) Spectrofluorometric analysis for MitoSOX Red fluorescence. Error bars represent the mean ± S.E.M. of three individual experiments. *($p < 0.05$) and **($p < 0.01$) are treatments significantly different from control (0.1% DMSO) or corresponding treatments. (B) Representative fluorescence microscopy for MitoSOX Red and MitoTracker Green fluorescence in MCF-7 cells (40× magnification).

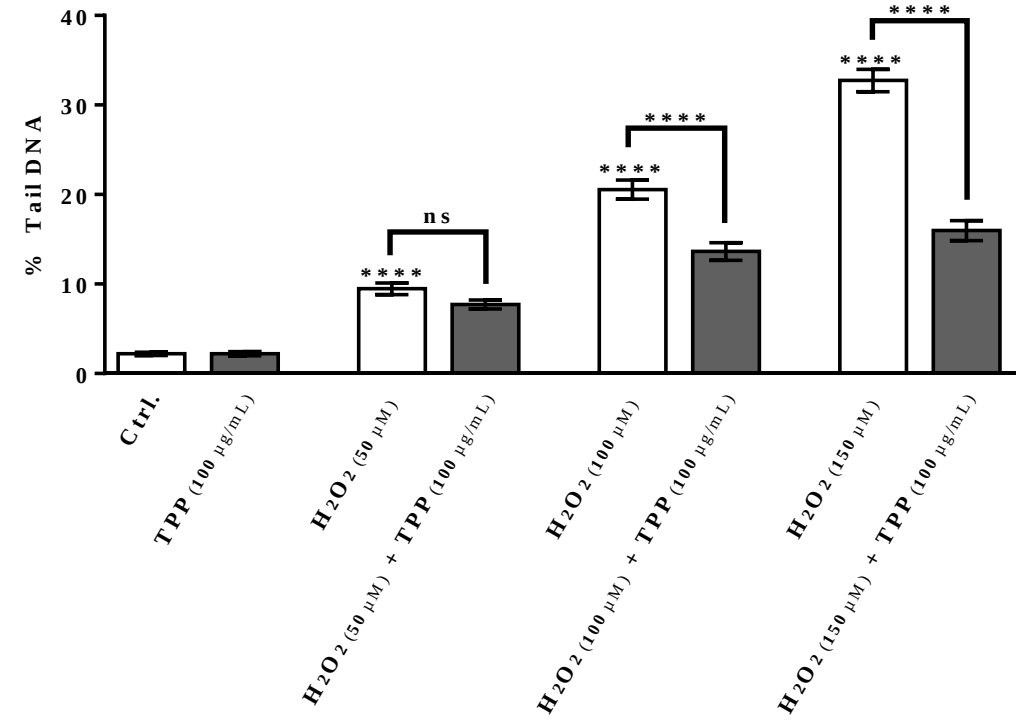
E2-induced DNA damage is inhibited by TPP in estrogen-dependent breast cancer cells.

Since DNA damage is a naturally expected consequence of cellular metabolism,[27] the possibility that E2-induced ROS production might increase DNA damage was considered. DNA damage was evaluated using the single cell gel electrophoresis (comet assay), which measures the percentage of tail DNA as an indicator of DNA damage. As shown in Figure 3.4.7A, E2 treatment caused an increase in % tail DNA, whereas TPP showed no evident effect on % tail DNA in MCF-7 cells. An increase in % tail DNA of E2 and the positive control H_2O_2 over DMSO-treated control was evident as early as 3 h after treatment and persisted for at least 24 h (p <0.0001) (Figure 3.4.7A). Intriguingly, co-treatment with TPP decreased E2-induced % tail DNA (p <0.0001) for both time periods. Furthermore, co-treatment of H_2O_2 with TPP decreased H_2O_2-induced % tail DNA in MCF-7 cells, in a concentration-dependent manner (Figure 3.4.7B). DNA damage by E2 and H_2O_2 treatments in MCF-7 cells was confirmed by fluorescence microscopy after staining the DNA with PI. As shown in Figure 3.4.7C, DMSO-treated control exhibited no effect on the nucleoid body. However, MCF-7 cells treated with 100 ng/mL E2 and 100 µM H_2O_2 showed typical comet formations. Interestingly, E2 and H_2O_2 co-treated with TPP did not show any evidence of DNA damage (Figure 3.4.7C). Altogether, these results show that E2 and H_2O_2 induces DNA damage in MCF-7 cells and this could be inhibited by treatment with TPP.

A


% Tail DNA
25
20
15
10
5
0
****
****
****
****
****
****
Ctrl. (0.1% DMSO)
TPP (100 µg/mL)
E2 (100 ng/mL)
E2 (100 ng/mL) + TPP (100 µg/mL)
Ctrl. (H2O2)
H2O2 (50 µM)
Ctrl. (0.1% DMSO)
TPP (100 µg/mL)
E2 (100 ng/mL)
E2 (100 ng/mL) + TPP (100 µg/mL)
Ctrl. (H2O2)
H2O2 (50 µM)
3 h
24 h

B


% Tail DNA
40
30
20
10
0
ns
****
****
****
****
****
Ctrl.
TPP (100 µg/mL)
H2O2 (50 µM)
H2O2 (50 µM) + TPP (100 µg/mL)
H2O2 (100 µM)
H2O2 (100 µM) + TPP (100 µg/mL)
H2O2 (150 µM)
H2O2 (150 µM) + TPP (100 µg/mL)

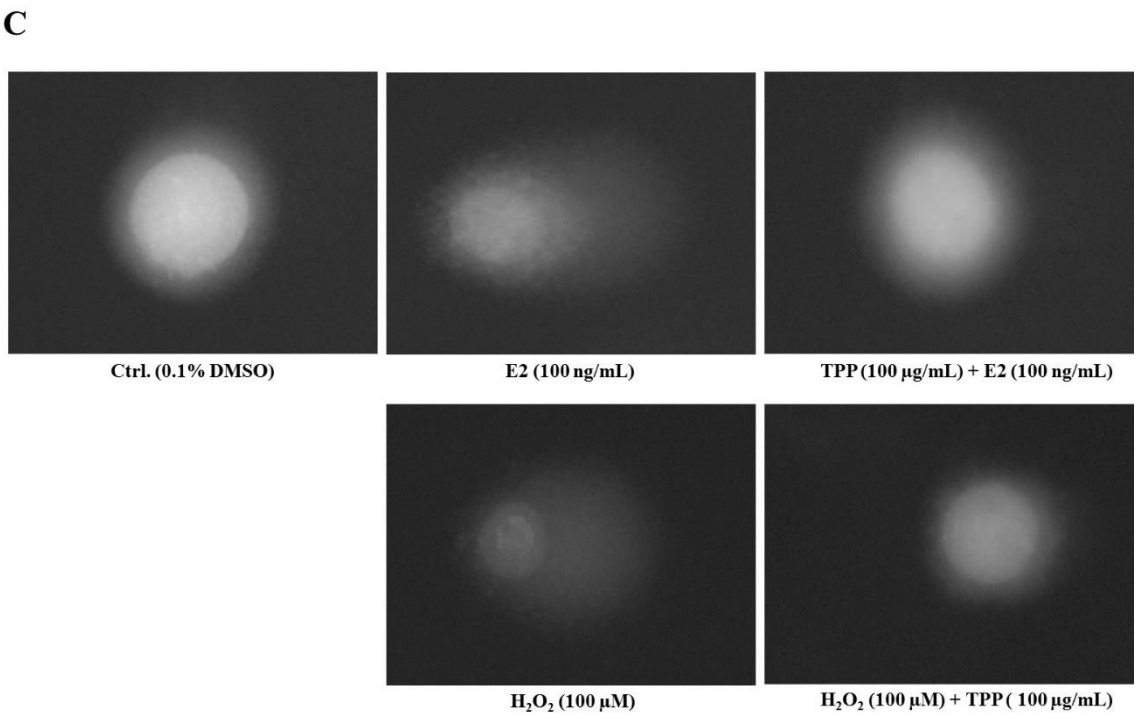


Figure 3.4.7 E2 increases DNA damage and co-treatment with TPP decreases E2-induced DNA damage in MCF-7 cells, determined by the comet assay. Cells were treated with (A) 100 µg/mL TPP, 100 ng/mL E2, E2 co-treatment with TPP, or 50 µM H_2O_2, on RT, for 3 h or 24 h; (B) 100 µg/mL TPP, 50 µM, 100 µM, 150 µM H_2O_2, or TPP co-treatments with H_2O_2, on RT, for 3 h; and then processed for alkaline comet assay. Error bars represent the mean ± S.E.M. of 300 determinations from three individual experiments. ****($p < 0.0001$) treatments are significantly different from control (0.1% DMSO) or corresponding E2 or H_2O_2 concentrations. (C) Representative comet formation images of MCF-7 cells for 24 h treatment.

3.5 Discussion

This is the first report of the inhibitory effect of mitochondria-targeted cation, TPP, on E2-induced responses in MCF-7 cells. Our results demonstrate a profound relationship between TPP and E2-induced cellular viability, growth, proliferation, mitochondrial bioenergetics, mitochondrial ROS production, and DNA damage suggesting a potential contribution of mitochondria in the development of estrogen-dependent breast cancer.

Given the 'classical' understanding that estrogens induce breast cancer cell proliferation and differentiation, and are considered risk factors for breast cancer development and progression for estrogen-dependent breast cancer cells, such as MCF-7, compared to estrogen independent breast cancer cells, such as MDA-MB231,[28] our results demonstrated positive relationships between E2 and cell viability, growth, DNA synthesis, mitochondrial bioenergetics, ROS production, mitochondrial ROS generation, and DNA damage in MCF-7 cells. Indeed, several studies have reported the estrogen-induced growth, DNA synthesis and proliferation, ROS production, and DNA damage for several estrogen-dependent cells.[7,22,29–32] In contrast, MDA-MB231 and MCF-10A show no correlation with these E2-induced endpoints.

TPP cation is generally considered biologically 'inert' and can slightly, but insignificantly, influence mitochondrial metabolism.[33] Our results showed that TPP concentrations have no notable effect on the three cell lines. However, TPP cation has shown to produce an uncoupling effect, which may affect the membrane potential and prevent fast production of ROS in mitochondria.[33,34]. TPP has also been shown to inhibit oxidative phosphorylation through inducing a negative effect on the respiratory chain

complexes, as well as on mitochondrial membrane potential and ATP synthesis.[17] Indeed, our data showed profound findings when E2-induced cellular activities are treated with TPP. E2 co-treated with TPP, dose-responsively and specifically, reduces mitochondrial and cellular activities in MCF-7 cells. E2-induced viability and growth demonstrated a remarkable decrease when co-treated with TPP. This suggests that TPP inhibition of E2-induced viability and growth could be associated with mitochondrial growth signaling and biogenesis. Estrogen-induced cell growth has been linked to mitochondrial signaling pathways that regulates progression of the cell cycle.[35] Since TPP has shown to disturb mitochondrial functions, the hypothesis that TPP inhibits E2-induced cell viability and growth by altering the mitochondrial signaling pathways involved in the progression of the cell cycle is intriguingly applicable.

Consistent with the above notion, TPP also inhibited E2-induced proliferation which was detected through BrdU incorporation into cellular DNA during cell proliferation in MCF-7 cells. In our model, cells were synchronized and held at the G1/S phase checkpoint by serum starvation and then transited toward the S phase by the addition of E2.[30] Indeed, E2 has shown to significantly increase DNA synthesis which was mediated by mitochondrial ROS signaling.[30] In this sense, TPP suppression of E2-induced DNA synthesis or S phase progression may relate, in part, to mitochondrial dynamics.

There are few reports that have investigated the effect of estrogen on $\Delta\Psi_m$. A recent report suggested that low concentration of E2 (10 nM ≈ 2.7 ng/mL) could change the $\Delta\Psi_m$ and increase the high $\Delta\Psi_m$ percentage in human spermatozoa.[36] Another report showed that E2 inhibits apoptotic proteins that mainly affect the $\Delta\Psi_m$ during apoptosis and result in

supporting the $\Delta\Psi_m$.[37] In contrast, Simpson et al. reported that high concentration of E2 (up to 100 μM ≈ 27 μg/mL) did not affect the $\Delta\Psi_m$ of human neuroblastoma cells.[38] However, since estrogen can increase the inner fluidity of the mitochondrial membrane, an increase in the $\Delta\Psi_m$ results from the increased electron transfer rate.[39] Also, our mitochondrial bioenergetics measurement data showed that E2 increased $\Delta\Psi_m$ in MCF-7 cells (Figure 3.4.4). Additionally, TPP, through its action at the $\Delta\Psi_m$, inhibited the E2-induced increase in $\Delta\Psi_m$. Therefore, the assumption that estrogen may have the ability to increase the mitochondrial membrane potential is very relevant and could be a key mechanism in the development of estrogen-dependent breast cancer.

ROS production in our estrogen-induced model was investigated, since it was suggested that the formation of ROS in mitochondria occurs at a high $\Delta\Psi_m$.[40] Our data shows that the presence of TPP decreases the E2-induced ROS formation in MCF-7 cells. To confirm the source of ROS generated by our E2-induced model, we localized ROS using a mitochondrial ROS indicator. Our results showed that a significant proportion of E2-induced ROS is mitochondrial in origin and the presence of TPP decreases the E2-induced mitochondrial superoxide, as a form of ROS, in MCF-7 cells. Several reports have suggested that E2 stimulates the formation of ROS in different cells, *in-vitro*. For instance, E2 has been shown to stimulate intracellular ROS production via mitochondrial signaling, which suggests a potential role of mitochondrial ROS as signal-transducing messengers in MCF-7 cells.[22] Indeed, estrogen exposure has been shown to trigger ROS production, mainly mitochondrial ROS, involving the growth signaling of MCF-7 and vascular endothelial cells.[10,30] In this regard, our results demonstrate that ROS formation is

consistent with $\Delta\Psi_m$ measurements, and therefore suggest that estrogen modulation of $\Delta\Psi_m$ may be involved, mechanistically, in the generation of ROS, or vice versa.

Since DNA damage and repair are important factors in the development of human cancers,[41] we investigated E2-induced DNA damage. It has been reported that E2 treatment induces DNA damage and increases γH2AX, an early marker of DNA double-strand breaks.[32] Our data showed that E2 induces DNA damage and the co-treatment with TPP inhibits E2-induced DNA damage, and therefore suggest the E2-induced mitochondrial ROS as a possible mechanism in the DNA damage of estrogen-dependent breast cancer.

In summary, our findings propose that TPP inhibition of E2-induced mitochondrial dynamics and metabolism hints for a possible mitochondrial function in cancer affected tissues. Further investigations are needed to clarify the exact mechanisms by which E2-induced activities are inhibited by mitochondria-targeted cation. By providing a foundation for the utility of mitochondria-targeted lipophilic cations, such as TPP, this work opens the way for the rational design of mitochondria-targeted drugs directed against estrogen-dependent breast tumors.

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3.7 Supplementary Data

Figure 3.4.1.S

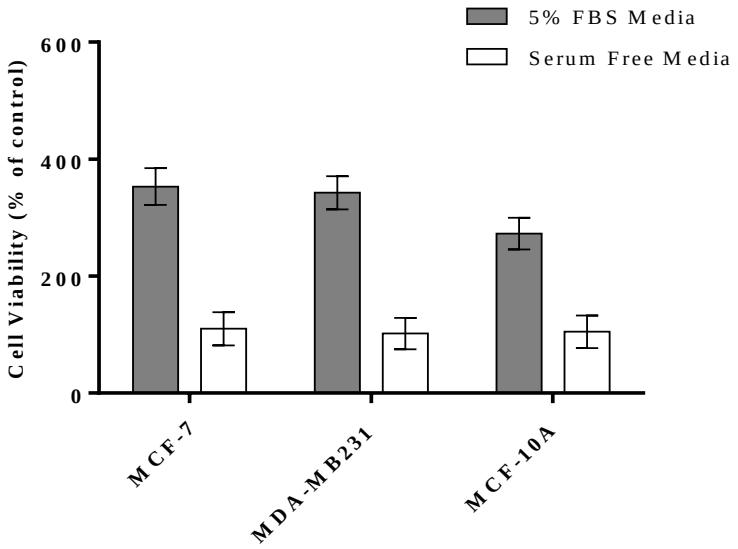
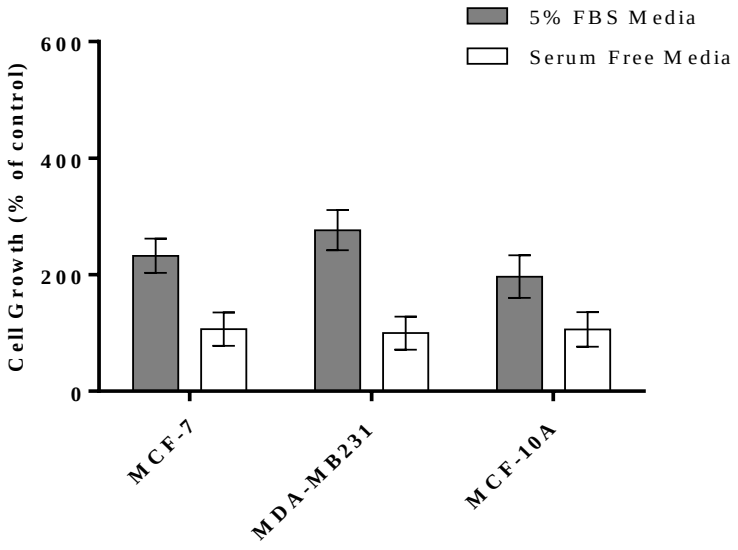


Figure 3.4.2.S



CHAPTER 4

ANTITUMOR ACTIVITY OF EXTRACT AND MITOCHONDRIA-TARGETED BIOCONJUGATE OF *ORIGANUM MAJORANA* IN BREAST CANCER

4.1 Abstract

Origanum majorana, commonly known as marjoram, is a perennial herb, which is widely used in the Middle East as a spice. It has been shown to possess extensive range of biological activity, including antioxidant, anti-inflammatory, and anti-tumor growth effects. Interestingly, the anticancer potential of *O. majorana* against breast cancer remains largely unexplored. The aim of this study was to evaluate the cytotoxic effects of *O. majorana* organic extract (OME) as well as a PEGylated bioconjugate of OME with triphenylphosphonium (P-OME-TPP) against human breast epithelial and cancer cell lines. Herein, the anticancer effect of *O. majorana* on three breast cell lines, MDA-MB231, MCF-7, and MCF-10A cells, was investigated. Triphenylphosphonium (TPP) cation was used to specifically deliver the bioconjugate to mitochondria. Determination of mitochondrial metabolic activity (cell viability) and cell density (cell growth) showed that OME blocked E2-induced cell viability and growth of breast cancer MCF-7 cells. Both OME and P-OME-TPP inhibited viability and growth of MDA-MB231 and MCF-7 cells, but no effect on normal breast epithelial MCF-10A cells was observed. Finally, the results also demonstrated that P-OME-TPP conjugate, compared to OME, was far more effective in exerting its cytotoxic effect through the inhibition of growth, and decrease in mitochondrial metabolic activity in both highly metastatic, triple negative MDA-MB-231 and estrogen-dependent MCF-7 breast cancer cells. These findings offer a new perspective on the potential of *O. majorana* extract to be developed as a new therapy against breast tumors.

4.2 Introduction

Breast cancer is the most common cancer in women and the second most common cancer among all cancers worldwide.[1] These high rates are probably due to the interaction of multiple environmental and biological factors. These interactions make breast cancer a heterogeneous disease, which give rise to therapeutic challenges such as disease resistance and recurrence, and the requirement for immensely toxic therapy. Indeed, up to 50% of patients are treated with hormonal therapy and chemotherapy; and of them almost 35% are resistant to current therapy.[2] Aggressive tumors are usually resistant to therapy; thus, treatments are not effective. The existing therapeutic agents used on aggressive tumors, such as chemotherapy, are extremely toxic.[3] There is an urgent need to develop effective and less toxic therapy against aggressive breast cancer. To successfully produce a better treatment with increased efficacy and low toxicity, it is necessary to select therapies based on the clinical and molecular characteristics of the tumor, of which there are at least three significant subtypes, based on gene expression profiles.[4] Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) receptor status is very important in categorizing breast tumors to successfully predict outcome and help in controlling of the disease development.[5]

Estrogens have a major role in breast tumor initiation and progression. Epidemiological and clinical evidences have associated the increased lifetime exposure to estrogens with high risk of developing breast cancer.[6–8] Nevertheless, the mechanisms involved in estrogen-induced breast carcinogenesis are still unclear. Experimentally, estrogens are considered breast carcinogens, as a result of their ability to initiate and

promote the development of cancer.[9–12] Precisely, ER-mediated signaling pathways are the most recognized pathways in estrogen carcinogenic mechanisms.[13,14] As most of breast cancer hormonal therapies are designed for the ER-positive cancer, they are mainly limited to anti-estrogenic agents targeting ER.[15] Selective estrogen receptor modulators (SERMs); such as Tamoxifen, and antiestrogens; such as ICI 182,780 (Fulvestrant) are commonly used in ER-positive tumor treatment.[15] Even though SERMs might prevent the growth of cancer due to their actions at the ER, other contributing mechanisms cannot be excluded, since these compounds also inhibit metabolism and redox cycling of estrogen and act as free radical scavengers.[16] Whereas, nearly 70% of newly diagnosed breast cancer patients have a positive expression of ER, approximately 50% of those patients develop resistance to most anti-estrogenic agents.[17] In addition, breast cancer characterized by lack of ER, PR, and overexpression of HER2, often called triple-negative breast cancer (TNBC), is typically associated with poor prognosis due to aggressive tumor phenotypes and current lack of effective and specific therapies.[18] The traditional chemotherapy drugs cause non-specific toxicity, deteriorate the patient's quality of life, weaken the immune system, and result in off-target effects on normal tissues.[19]

Mitochondria are important subcellular organelles that play a critical role in cellular metabolism, calcium homeostasis, redox signaling pathways, apoptosis and cell death.[20] Dysfunctional mitochondria are common among various diseases and specifically to cancer tumor cells, which is explained by the conversion of the source of metabolic energy from oxidative phosphorylation to active glycolysis and increased ROS formation.[21] Although estrogen may be involved in breast carcinogenesis through increasing the mitochondrial-

derived ROS, the precise mitochondrial mechanisms for estrogen-induced breast carcinogenesis are still unclear.[22] For example, the most potent endogenous estrogen, 17-β-estradiol (E2), was shown to promote DNA synthesis in breast cancer cells via mitochondrial oxidant signaling.[22] Thus, mitochondrial targeting strategy has drawn attention, recently, as a cancer therapeutic strategy and several investigations in this area have been reported.[23]

Accumulation of mitochondria-specific drugs is necessary in optimizing tumor-targeted therapeutic agents. In this case, the triphenylphosphonium (TPP) cation have been used to deliver biologically active compound and molecules; such as antioxidants, to mitochondria, which indicates the inclusive utility of this approach for mitochondria targeting.[24] TPP is a lipophilic cation that possess the property of being lipid-soluble, despite its total positive charge. This property allows TPP to cross the cell membrane phospholipid bilayers, as well as the mitochondrial membrane.[25] The large membrane potential, approximately -180 mV, across the mitochondrial inner membrane is used to specifically deliver molecules to mitochondria. TPP conjugated to a therapeutic molecule will pass the cell membrane using the lipid-soluble property. The uptake into the cytoplasm across the plasma membrane is driven by the plasma membrane potential (-30 to -60 mV, internal).[25] From the cytoplasm, the TPP conjugates are further accumulated into the mitochondria, which is driven by the mitochondrial membrane potential ($\Delta\Psi_m$). The accumulation of TPP conjugates in the cytoplasm and mitochondria is about 5-10 and 100-500 fold, respectively, compared to the extracellular environment .[25]

An increasing number of studies have shown that phytochemicals are important with regards to their cancer chemopreventive properties and their ability to decrease tumor growth.[26] Phytochemicals can regulate several pathways used by cancer cells in the processes of cell growth and proliferation, survival, angiogenesis, invasion, metastasis, and apoptosis.[27] *Origanum majorana*, commonly known as marjoram, is worldwide perennial herb. It is utilized as a spice, flavoring agent, and has an ancient culinary and medicinal use.[28] It has been used as a traditional medicine for various illnesses such as chest infections, cough, sore throat, rheumatism, cardiovascular diseases, abdominal pain, insomnia, anxiety disorder, as well as skin care.[29,30] Several studies have reported that *O. majorana* is rich in phenolic compounds, which possess the capacity to scavenge free radicals and exhibit a strong antioxidant property.[31] A few studies have reported the anticancer potential of *O. majorana*; however, it remains largely unexplored.[32]

To specifically target the tumor with enhanced efficacy and reduced non-targeted toxicity, small molecule bioconjugation therapy usually used due to their unique physical and biological properties.[33] Polyethylene glycol (PEG), is an amphiphilic polymer, provides electrostatic and steric stabilization, and a longer circulation half-life *in vivo* as well as functional-end groups for the attachment of targeting ligands.[33] PEGylation of anticancer compounds is to protect the molecules from destruction by the reticuloendothelial system (RES) and, therefore, increase circulation time and drug accumulation in the tumors.[34] In this study, *O. majorana* extract (OME) was used to synthesize mitochondria-targeted bioconjugate of *O. majorana* (P-OME-TPP) and its cytotoxicity in 17β-estradiol (E2) treated human breast epithelial and cancer cell lines was

evaluated. The antitumor property of OME and P-OME-TPP was measured using cell viability and cell growth assays.

4.2 Materials and methods

4.2.1 Chemicals and reagents.

17β-Estradiol, (4-carboxybutyl) triphenylphosphonium (TPP) bromide, dimethylsulfoxide (DMSO), 3(4,5-dimethylthiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT), sulforhodamine B (SRB), trichloroacetic acid (TCA), poly-(ethylene glycol)-4000 [PEG_{4000}; powder; MW 3500-4500], and block copolymer surfactant pluronic P123 were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA), unless otherwise specified.

4.2.2 Preparation of the *O. majorana* ethanolic extract (OME).

O. marjorana was obtained from a herbal store located in the Madinah region of Saudi Arabia. The extract was prepared as described by Dhaheri et al.[32] Briefly, 5.0 g of the dried leaves were ground to a powder using a porcelain pestle and mortar. The powder was suspended in 100 mL of 70% (v/v) absolute ethanol and the mixture kept in the dark for 72 h at 4 °C without stirring. Using a sintered glass funnel, the mixture was filtered and the filtrate then evaporated to dryness using a rotary evaporator at RT. The resulting green powder residue was kept under vacuum for 3 h and its mass then recorded.

4.2.3 Synthesis of P-OME-TPP bioconjugate.

The bioconjugate was synthesized according to Bhattacharya et al. with minor modification.[35] Briefly, 120 mg of dry OME was added in 20 mL of ethanol. Next, 20 mg of surfactant (P123) was added to the mixture and stirred briefly. Then, 20 mg of TPP was added to the mixture and stirred for 15 min. PEG was prepared by homogenizing 10 g of PEG_{4000} in 50 mL of deionized water, using ice-bath sonication for 20-30 min. When the PEG was completely dissolved, the OME-TPP mixture was drop-wise mixed with PEG under sonicating condition. When the addition was completed, the mixture was kept under sonicating condition for another 30 min, which was followed by an overnight stirring at RT. Next day, the organic solvent (ethanol) present in suspension was evaporated by a rotary evaporator and a solution of P-OME-TPP conjugate was obtained.

4.2.4 Characterization of P-OME-TPP bioconjugate by UV/Vis Spectrophotometry.

PEG, TPP, P123, OME, and P-OME-TPP bioconjugate were characterized by measuring the absorbance by UV/Vis Spectrophotometer at range of 200-450 nm.

4.2.5 Cell culture.

The estrogen-dependent breast cancer (MCF-7), metastatic breast cancer (MDA-MB231), and normal epithelial breast (MCF-10A) cells were routinely cultured in phenol red DMEM-F12 media (1:1) supplemented with 5% (v/v) fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin (complete media) at 37 °C in a 5% CO_2 humidified incubator. For experimental purposes, culture media were changed to starvation

media (serum free media + antibiotics) and allowed to incubate for 48 h prior to commencement of all experiments, unless otherwise indicated.

4.2.6 Cell viability.

MTT was used to determine cell viability according to the manufacturer's protocol.[36] In brief, MCF7, MCF-10A, and MDA-MB231 cells were seeded in three separate 96-well plates (approx. 7500 cells/well) overnight in complete media and serum starved for 48 h prior to treatment with OME, P-OME-TPP, and OME with E2 for 24 h or 48 h. Then, 20 µL of (5 mg/mL) MTT was added to each well. After incubation for 3.5 h at 37 °C, the media was carefully removed and 100 µL of MTT solvent (100% DMSO) was added to each well. The plates were then covered with aluminium foil and agitated on shaker for 15 min. Absorbance was recorded at 590 nm (reference λ at 620 nm) on a Tecan Genios microplate reader.

4.2.7 Cell growth.

SRB was used to determine cell growth as described by Skehan et al.[37] Briefly, cells were seeded in three separate 96-well plates (approx. 7500 cells/well) for 24 h in complete media and serum starved for 48 h prior to adding the various cell treatments for 24 h or 48 h. The cells were then fixed with 50% TCA solution, layered on top of the existing medium, and chilled to 4 °C. After 1 h of incubation at 4 °C, the plates were rinsed four times with distilled water. Then 100 µL of 0.4% (w/v) SRB was added to cover the bottom of the well and allowed to stain for 20 min. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris

(hydroxymethyl) aminomethane]. Absorbance was determined at 590 nm (reference λ at 620 nm) on a Tecan Genios microplate reader.

4.2.8 Statistical Analysis.

Statistical analyses was performed using GraphPad Prism, version 6 (GraphPad, CA, USA). The data was evaluated using one-way ANOVA and Tukey's multiple comparisons tests to compare the values between groups. The level of statistical significance was set at $P < 0.05$.

4.3 Results

OME inhibits E2-induced cellular viability of estrogen-dependent breast cancer cells.

The time- and dose-dependent effect of E2 (100 pg/mL and 100 ng/mL) and co-treatment of OME with E2 (300 µg/mL) on the viability of MCF-7, MDA-MB231, and MCF-10A cells in serum-free conditions for 24 h and 48 h was tested. As shown in Figure 4.3.1, a significant time- and dose-dependent increase in cell viability was observed for both E2 100 pg/mL ($p < 0.0001$) and E2 100 ng/mL ($p < 0.0001$) for MCF-7 cells for both periods 24 h and 48 h (Figure 4.3.1A). This increase was not observed for MDA-MB231 (Figure 4.3.1B) nor MCF-10A (Figure 4.3.1C) cells. This confirms the estrogen-dependency of MCF-7 cells viability. Interestingly, the OME treatment had a significant time and dose-dependent decrease in E2-induced cell viability of MCF-7. The cell viability of E2 (100 pg/mL and 100 ng/mL) treatments were significantly inhibited by co-treatment with OME ($p < 0.0001$). The serum-free conditions used in these experiments were optimal for

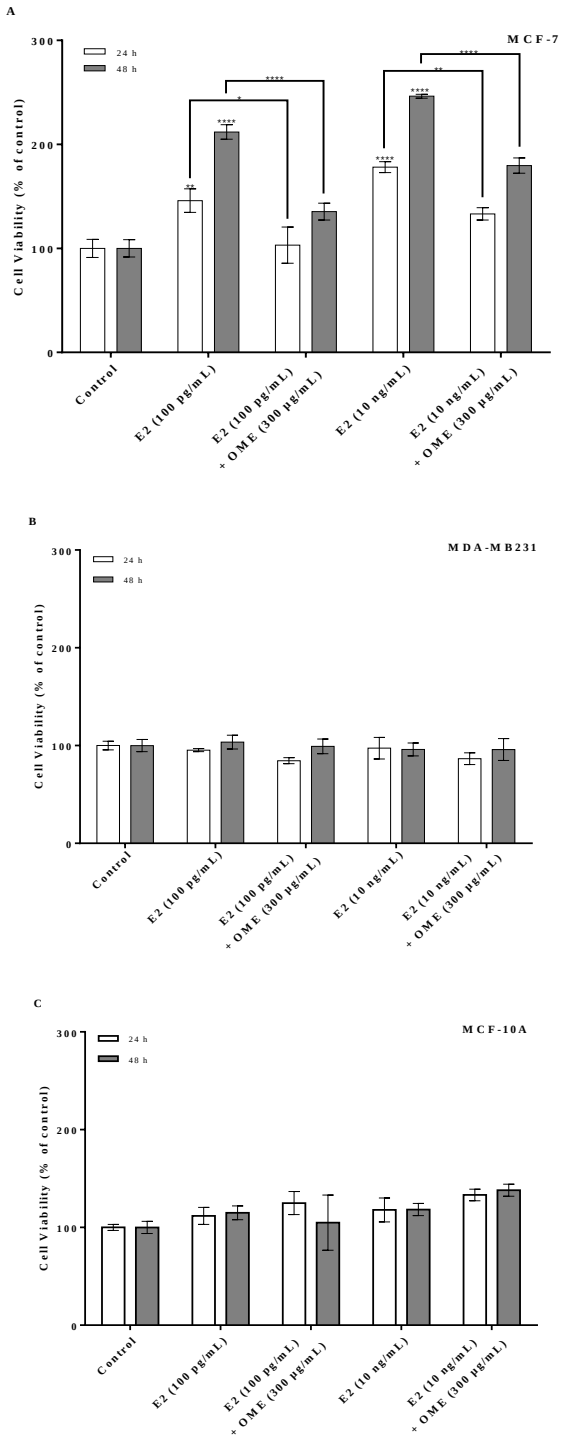


Figure 4.3.1 OME inhibited E2-induced MCF-7 cell viability, determined by MTT assay. Cell viability for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatment with E2, OME, or E2 co-treatment with OME for 24 h and 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments with control (ethanol) set as 100% cell viability. * ($p < 0.05$), ** ($p < 0.01$), and **** ($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

observing E2-induced effect on breast cancer cells, since serum deprivation synchronizes cells in the G0/G1 phase of the cell cycle.[38]

OME inhibits E2-induced cell growth of estrogen-dependent breast cancer cells.

To confirm the inhibition of MCF-7 cell viability by co-treatment of OME with E2, SRB assay was used to determine the cell growth for the three cell lines. A significant time- and dose-dependent increase in cell growth was noticed for both E2 concentrations ($p < 0.0001$) on MCF-7 cell (Figure 4.3.2A). However, this was not noticed for MDA-MB231 (Figure 4.3.2B) nor MCF-10A (Figure 4.3.2C) cells. Intriguingly, OME had a significant time- and dose-dependent inhibition in E2-induced cell growth of MCF-7 cells. The cell growth of E2 (100 pg/mL and 100 ng/mL) treatments were significantly inhibited by co-treatment with OME ($p < 0.0001$). These results demonstrated and confirmed that OME inhibits E2-induced cell viability and growth of MCF-7 cells.

Confirmation of conjugation of P-OME-TPP bioconjugate by UV/Vis Spectrophotometry.

The presence of the OME in PEG conjugate was evaluated by UV-Visible spectroscopy. The UV-Visible spectra of all conjugate components including poly-(ethylene glycol) (PEG_{4000}), triphenylphosphonium (TPP), surfactant (P123), *O. majorana* extract (OME), and bioconjugate of *O. majorana* (P-OME-TPP) are showed in Figure 4.3.3. The PEG_{4000} showed no absorbance peak (Figure 4.3.3A), whereas TPP showed absorbance peak at 202 nm (Figure 4.3.3B), P123 at 204 nm (Figure 4.3.3C), OME at 208 nm, 287 nm, and 328 nm (Figure 4.3.3D), and P-OME-TPP at 225 nm, 268 nm, 275 nm and 329 nm (Figure

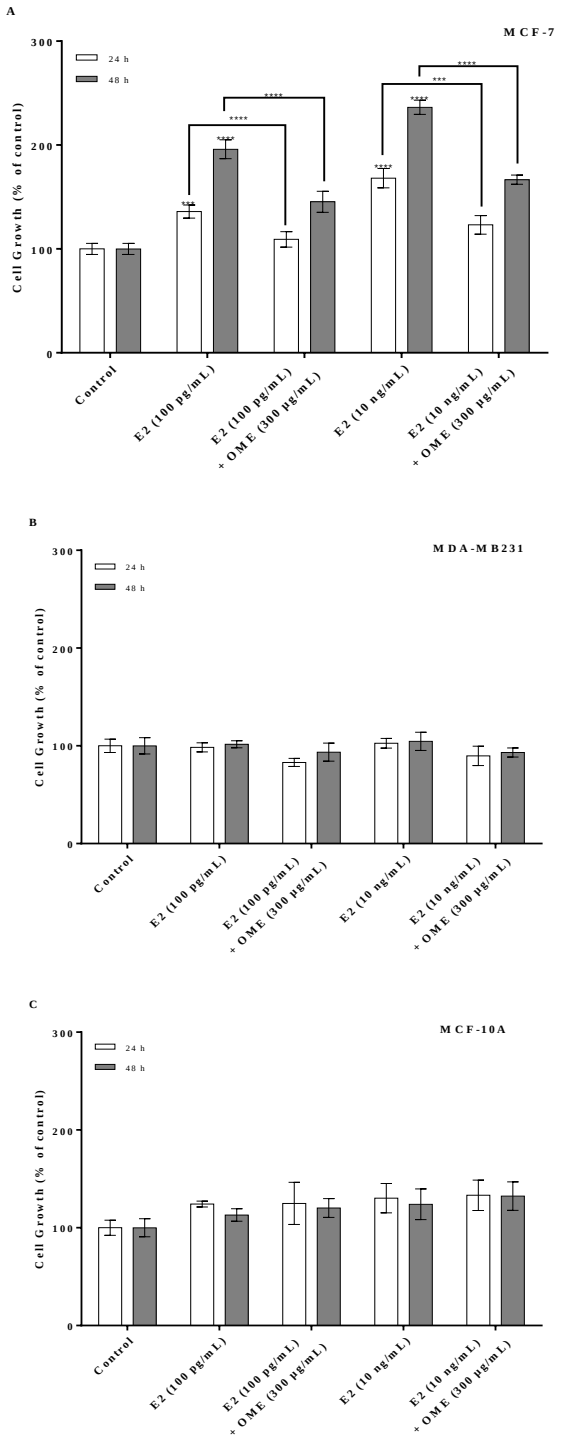


Figure 4.3.2 OME inhibited E2-induced MCF-7 cell growth, determined by SRB assay. Cell growth for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatment with E2, OME, or E2 co-treatment with OME for 24 h and 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments with control (ethanol) set as 100% cell growth. ***($p < 0.001$) and ****($p < 0.0001$) treatments are significantly different from control or corresponding E2 concentrations.

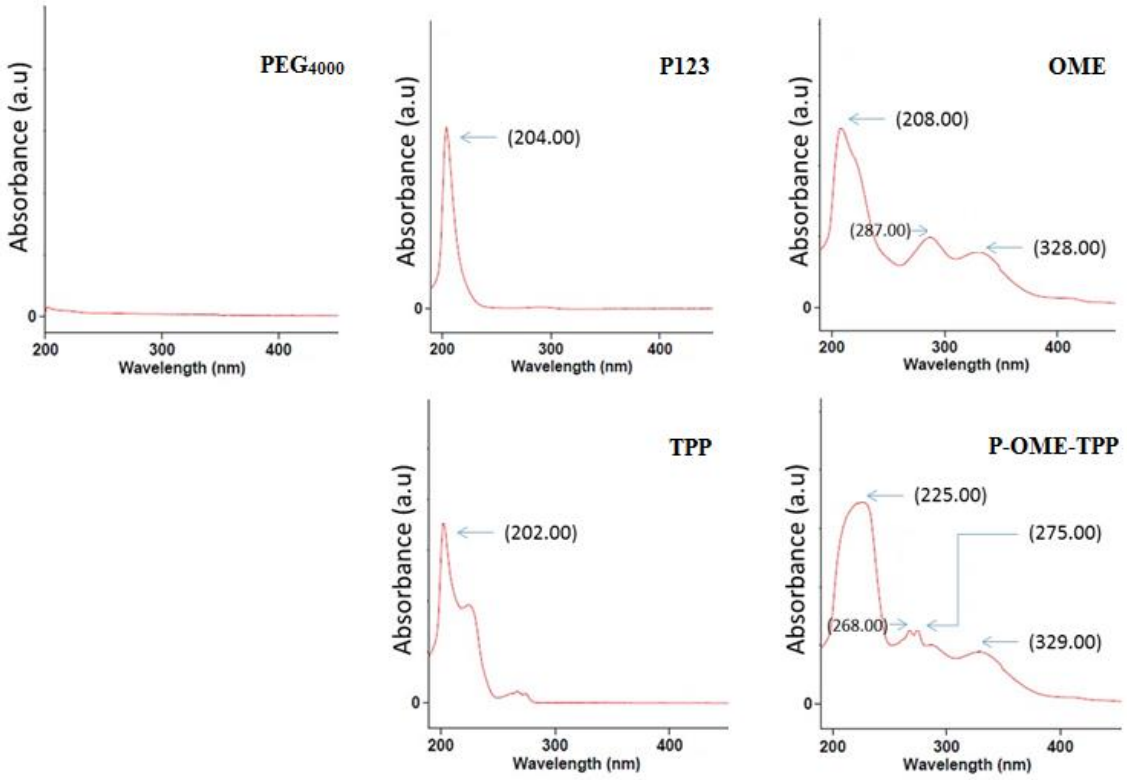


Figure 4.3.3 Analysis of P-OME-TPP bioconjugate components determined via UV/Vis Spectrophotometry. PEG, TPP, P123, OME, and P-OME-TPP were characterized by measuring the absorbance by UV/Vis Spectrophotometer at range of 200-450 nm. The absorbance peaks of all P-OME-TPP components were compared with P-OME-TPP peaks to identify new emerging peaks as a sign successful conjugation.

4.3.3E). The new absorbance peaks showed at 225 nm, 268 nm, 275 nm and 329 nm for P-OME-TPP indicated the presence of TPP, P123, and OME within the PEGylated bioconjugate, thereby confirming the proper preparation of the bioconjugate of *O. majorana* (P-OME-TPP).

OME and P-OME-TPP treatment inhibit the cellular viability of estrogen-dependent breast cancer (MCF-7) and metastatic breast cancer (MDA-MB231) cells.

Initially, the dose- and time-dependent effect OME and P-OME-TPP on the viability of MCF-7, MDA-MB231, and MCF-10A cells in serum-free conditions was tested. As shown in Figure 4.3.4, both MCF-7 and MDA-MB231 treated with OME had a significant time- and dose-dependent decrease in their viability. However, MCF-10A treated with OME did not influence cell viability. MCF-7 cell viability was significantly inhibited by OME concentrations of 300 µg/mL ($p < 0.001$), 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. Also, it was significantly inhibited by OME concentrations of 100 µg/mL, 300 µg/mL, 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 48 h period (Figure 4.3.4A). MDA-MB231 cell viability was also significantly inhibited by OME concentrations of 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. It was also significantly inhibited by OME concentrations of 100 µg/mL ($p < 0.01$), 300 µg/mL, 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 48 h period (Figure 4.3.4B). MCF-10A did not show significant decrease in viability by OME concentrations for both 24 h and 48 h periods (Figure 4.3.4C).

Furthermore, both MCF-7 and MDA-MB231 cells treated with P-OME-TPP had a significant time- and dose-dependent decline in their viability (Figure 4.3.4A and B). However, this decrease was not observed for MCF-10A cells. MCF-7 cell viability was

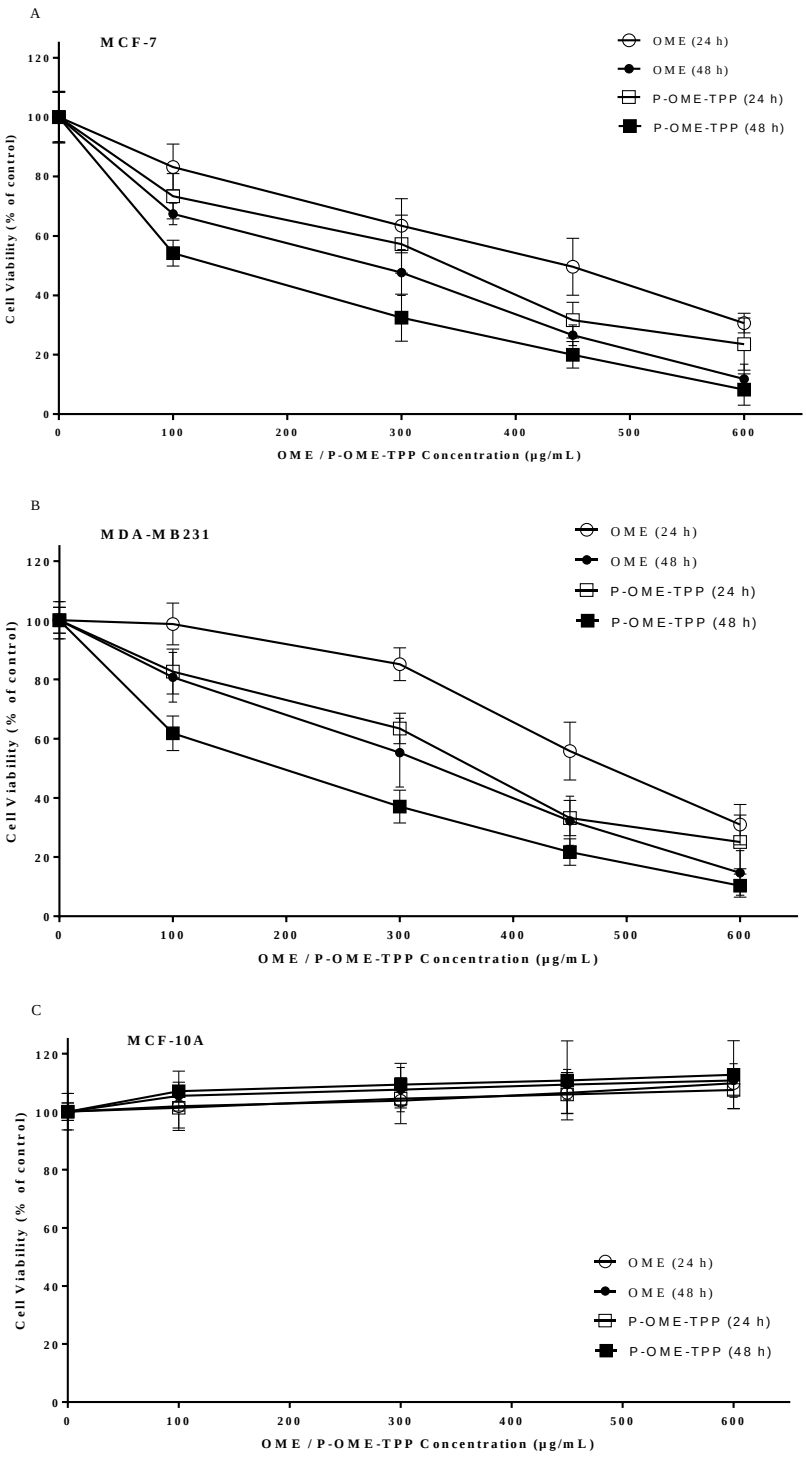


Figure 4.3.4 OME and P-OME-TPP bio-conjugate decreased MDA-MB231 and MCF-7 cell viability, determined by MTT assay. Cell viability for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatment with OME or P-OME-TPP for 24 h and 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments with control (ethanol) set as 100% cell viability.

significantly inhibited by P-OME-TPP concentrations of 100 µg/mL ($p < 0.01$), 300 µg/mL ($p < 0.001$), 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. Also, it was significantly inhibited by all P-OME-TPP concentrations ($p < 0.0001$) for the 48 h period (Figure 4.3.4A). For MDA-MB231, P-OME-TPP significantly inhibited cell viability of MDA-MB213 at 100 µg/mL ($p < 0.05$), 300 µg/mL ($p < 0.001$), 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. Also, MDA-MB231 cell viability was significantly inhibited by all P-OME-TPP concentrations ($p < 0.0001$) for the 48 h period (Figure 4.3.4B). MCF-10A did not display any significant inhibition in viability by P-OME-TPP concentrations for both 24 h and 48 h periods (Figure 4.3.4C). Also, P-OME-TPP conjugate individual components, consisted of PEG, P123, and TPP, did not show any significant effect on all treated cell lines (Figure 4.3.1S).

Compared to OME treatment, P-OME-TPP significantly inhibited the viability of MCF-7 with a concentration as low as 100 µg/mL ($p < 0.01$), whereas OME did not affect the viability at the same concentration for the 24 h period. Also, the half maximal inhibitory concentration (IC_{50}) of cell viability was lower for P-OME-TPP (276.6 µg/mL for 24 h and 123.8 µg/mL for 48 h) compared to OME (400 µg/mL for 24 h and 205.8 µg/mL for 48 h) (Table 4.3.1). Moreover, P-OME-TPP significantly inhibited the viability of MDA-MB231 with concentrations as low as 100 µg/mL ($p < 0.05$) and 300 µg/mL ($p < 0.001$), whereas OME did not show a significant effect on the viability at the same concentration for the 24 h period. Also, the IC_{50} of cell viability was lower for P-OME-TPP (337.5 µg/mL for 24 h and 158.6 µg/mL for 48 h) compared to OME (481 µg/mL for 24 h and 289.6 µg/mL for 48 h) (Table 4.3.1).

	Treatments / Calculated values	OME (24 h)	P-OME-TPP (24 h)	OME (48 h)	P-OME-TPP (48 h)
MCF-7	**IC50** (µg/mL)	400.2	276.6	205.8	123.8
	R square	0.9496	0.9073	0.899	0.9368
MDA-MB231	**IC50** (µg/mL)	481	337.5	289.6	158.6
	R square	0.9996	0.9363	0.9463	0.9517

Table 4.3.1 OME and P-OME-TPP assessment of IC_{50} for MCF-7 and MDA-MB231 cells viability.

OME and P-OME-TPP inhibited cell growth of MCF-7 and MDA-MB231 cells.

To confirm the inhibition of MCF-7 and MDA-MB231 cell viability by OME and P-OME-TPP, SRB assay, which detects cellular protein, was used to determine the cell growth for the three cell lines. The dose- and time-dependent effect of OME and P-OME-TPP on the growth of MCF-7, MDA-MB231, and MCF-10A cells in serum-free conditions was tested. As shown in Figure 4.3.5, both MCF-7 and MDA-MB231 treated with OME had a significant time- and dose-dependent decrease in their growth. However, this decrease was not observed for MCF-10A cells. MCF-7 cell growth was significantly inhibited by OME concentrations of 100 µg/mL ($p < 0.05$), 300 µg/mL ($p < 0.001$), 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. Also, it was significantly inhibited by all OME concentrations ($p < 0.0001$) for the 48 h period (Figure 4.3.5A). MDA-MB231 cell growth was also significantly inhibited by OME concentrations of 300 µg/mL ($p < 0.001$), 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. It was also significantly inhibited by all OME concentrations ($p < 0.0001$) for the 48 h period (Figure 4.3.5B). MCF-10A did not show a significant decrease in growth by OME concentrations for both 24 h and 48 h periods (Figure 4.3.5C).

Moreover, both MCF-7 and MDA-MB231 treated with P-OME-TPP had a significant time- and dose-dependent decrease in their growth (Figure 4.3.5A & B). However, this was not noticed in MCF-10A treated with P-OME-TPP. MCF-7 cell growth was significantly inhibited by P-OME-TPP concentrations of 100 µg/mL ($p < 0.001$), 300 µg/mL ($p < 0.001$), 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. Also, it was significantly inhibited by all OME concentrations ($p < 0.0001$) for the 48 h period (Figure

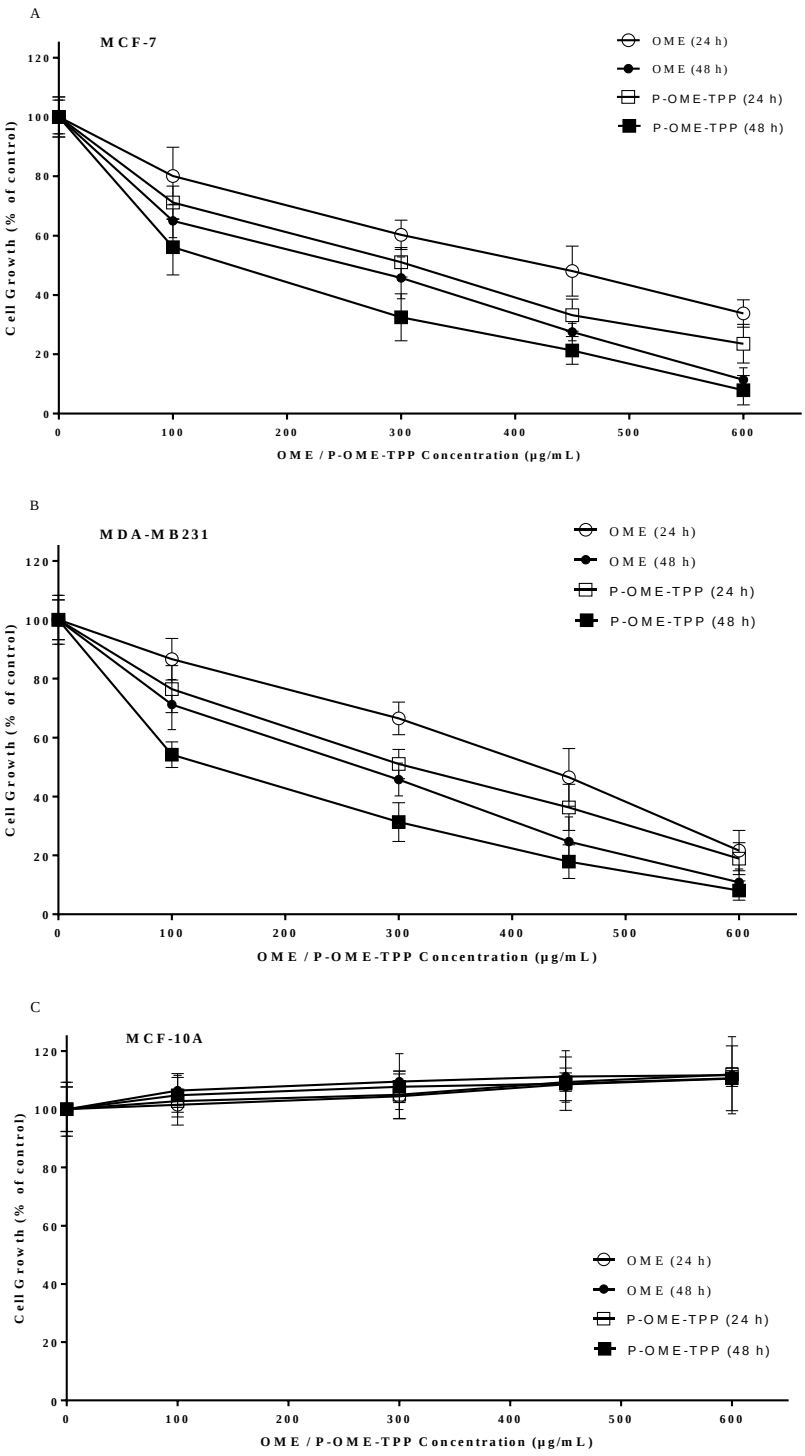


Figure 4.3.5 OME and P-OME-TPP decreased MDA-MB231 and MCF-7 cell growth, determined by SRB assay. Cell growth for (A) MCF-7; (B) MDA-MB231; (C) MCF-10A. Cells were cultured for 24 h in complete media then serum starved for 48 h prior to treatment with OME or P-OME-TPP for 24 h and 48 h. Results are expressed as the mean ± S.E.M. of three independent experiments with control (ethanol) set as 100% cell growth.

4.3.5A). For MDA-MB231, P-OME-TPP significantly inhibited cell growth at 100 µg/mL ($p < 0.01$), 300 µg/mL, 450 µg/mL and 600 µg/mL ($p < 0.0001$) for the 24 h period. Also, MDA-MB231 cell growth was significantly inhibited by all P-OME-TPP concentrations ($p < 0.0001$) for the 48 h period (Figure 4.3.5B). MCF-10A had no significant inhibition in viability by OME concentrations for both 24 h and 48 h periods (Figure 4.3.5C). Moreover, P-OME-TPP conjugate individual constituents, contained PEG, P123, and TPP, did not show any substantial effect on all treated cell lines (Figure 4.3.2S).

Additionally, the IC_{50} of MCF-7 cell growth was lower for P-OME-TPP (251.7 µg/mL for 24 h and 131.2 µg/mL for 48 h) compared to OME (387.9 µg/mL for 24 h and 192.7 µg/mL for 48 h) (Table 4.3.2). Furthermore, P-OME-TPP significantly inhibited the growth of MDA-MB231 with a concentration as low as 100 µg/mL ($p < 0.01$), whereas OME did not affect the growth at the same concentration for the 24 h period. The IC_{50} of cell growth was also lower for P-OME-TPP (271.9 µg/mL for 24 h and 122.1 µg/mL for 48 h) compared to OME (389.7 µg/mL for 24 h and 212.9 µg/mL for 48 h) (Table 4.3.2). Altogether, these results confirmed the cytotoxic effect of OME and P-OME-TPP on MCF-7 and MDA-MB231 cells.

	Treatments / Calculated values	OME (24 h)	P-OME-TPP (24 h)	OME (48 h)	P-OME-TPP (48 h)
MCF-7	**IC50** (μg/mL)	387.9	251.7	192.7	131.2
	R square	0.9688	0.9562	0.8927	0.9398
MDA-MB231	**IC50** (μg/mL)	389.7	271.9	212.9	122.1
	R square	0.9403	0.9556	0.9367	0.9489

Table 4.3.2 OME and P-OME-TPP assessment of IC50 for MCF-7 and MDA-MB231 cells growth.

4.4 Discussion

In the present study, we have shown for the first time that the *O. majorana* ethanolic extract was able to inhibit estrogen-induced viability and growth of MCF-7 cells in a time- and concentration-dependent manner. We have also demonstrated that the P-OME-TPP bioconjugate compared to OME was far more effective in exerting its cytotoxic effect through the induction of growth arrest and inhibition of mitochondrial metabolic activity which resulted in cell death of MCF-7 and the highly metastatic triple negative MDA-MB-231 cells, in a time- and concentration-dependent manner.

In fact, estrogens are considered risk factors for breast cancer development and progression for estrogen-dependent cell, such as MCF-7, compared to estrogen independent breast cancer cells, such as MDA-MB231, through the induction of cell proliferation and differentiation.[39] Indeed, estrogen-induced growth, DNA synthesis, proliferation, and ROS production have been reported for several estrogen-dependent cells.[38,40–44] Noteworthy, mitochondrial signaling pathways involved in the progression of cell cycle has been reported to regulate estrogen-induced cell growth.[45] Our results corroborate previous studies that demonstrated positive relationships between E2 and cell viability and growth in MCF-7 cells. On the contrary, MDA-MB231 and MCF-10A show no correlation with these E2 induced endpoints.[22,38,40,45]

Inhibition of cell cycle progress and induction of apoptosis and cell death are essential mechanisms of the traditional anti-cancer treatments. Several natural compounds have been reported to possess chemopreventive properties via these two mechanisms.[46–49] For example, *O. majorana* is reported to exhibit cytotoxic, membrane and DNA damaging

effects on human lung cancer cells.[50] Moreover, Abdel-Massih et al. reported that *O. majorana* extracts exhibit anti-proliferative effects and high antioxidant activity on human lymphoblastic leukemia cells.[51] Our results showed profound findings when E2-induced cellular activities are co-treated with OME. E2 co-treated with OME, time- and concentration-responsively, inhibits cellular activities in MCF-7 cells. E2-induced viability and growth are remarkably decreased when co-treated with OME. This suggests that the anti-proliferative effect of OME, by induction of apoptosis,[51] could inhibit E2-induced viability and growth.

There is a growing interest in targeted therapy using multiple bioactive and delivery molecules affecting several targets and/or pathways with enhanced efficacy and reduced non-targeted toxicity.[33] One of these approaches is the small molecule bioconjugation which could be achieved by PEGylation of anticancer compounds and delivery molecules to protect the molecules from destruction and increased circulation time and bioconjugate accumulation in the tumors.[34] Thus, we have successfully PEGylated OME with TPP, to specifically deliver the bioconjugate to mitochondria,[24] and tested it on MFC-7, MDA-MB231, and MCF-10A cells. Our results showed interesting findings when cells are treated with OME and P-OME-TPP. First, we demonstrated for the first time that *O. majorana* possesses potent anti-proliferative effects against the estrogen-dependent breast cancer cell line, MCF-7. In fact, *O. majorana* extract, time- and concentration-responsively, inhibits the viability and growth of MCF-7 cells. Indeed, Al Dhaheri et al. reported that *O. majorana* extract shows anticancer activity through inducing cell cycle arrest and apoptosis of the highly proliferative and invasive MDA-MB231 breast cancer cell line.[32] They also

reported that *O. majorana* exhibits anti-metastatic and anti-tumor growth activities by inhibiting NFκB signaling and nitric oxide production of MDA-MB231 cells.[52] Similarly, we showed that OME, time- and concentration-dependently, inhibits the viability and growth of MDA-MB231 cells. In contrast, MCF-10A cells treated with OME show no significant change regarding their viability and growth.

Mitochondria play a major role in apoptosis and cell death as well as regulating cell growth and proliferation, which make them a potential target in cancer therapy.[20] Herein, we demonstrate that the bioconjugate of *O. majorana* has potent cytotoxic effects against MCF-7 and MDA-MB231 cells. Indeed, our results showed that P-OME-TPP was much more effective regarding its cytotoxicity compared to OME in both MCF-7 and MDA-MB231, according to the calculated IC_{50}. This suggests that the inhibitory mechanism of P-OME-TPP could be associated with mitochondria by altering the mitochondrial signaling pathways involved in cell growth and proliferation.

In summary, this study clearly demonstrated, for the first time, that *O. majorana* extract was able to inhibit estrogen-induced viability and growth of MCF-7 cells. In addition, OME possesses cytotoxic effects against MCF-7 and MDA-MB-231 cells through the inhibition of cell viability and growth. Our results also demonstrated that P-OME-TPP conjugate, compared to OME, was far more effective in exerting its cytotoxic effect through the inhibition of cell growth and viability in both highly metastatic, triple negative MDA-MB231 and estrogen-dependent MCF-7 breast cancer cells. Thus, our findings offer a new perspective on the potential of *O. majorana* extract to be developed as a promising chemopreventive and new therapy against breast tumors.

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4.6 Supplementary Data

Figure 4.3.1S

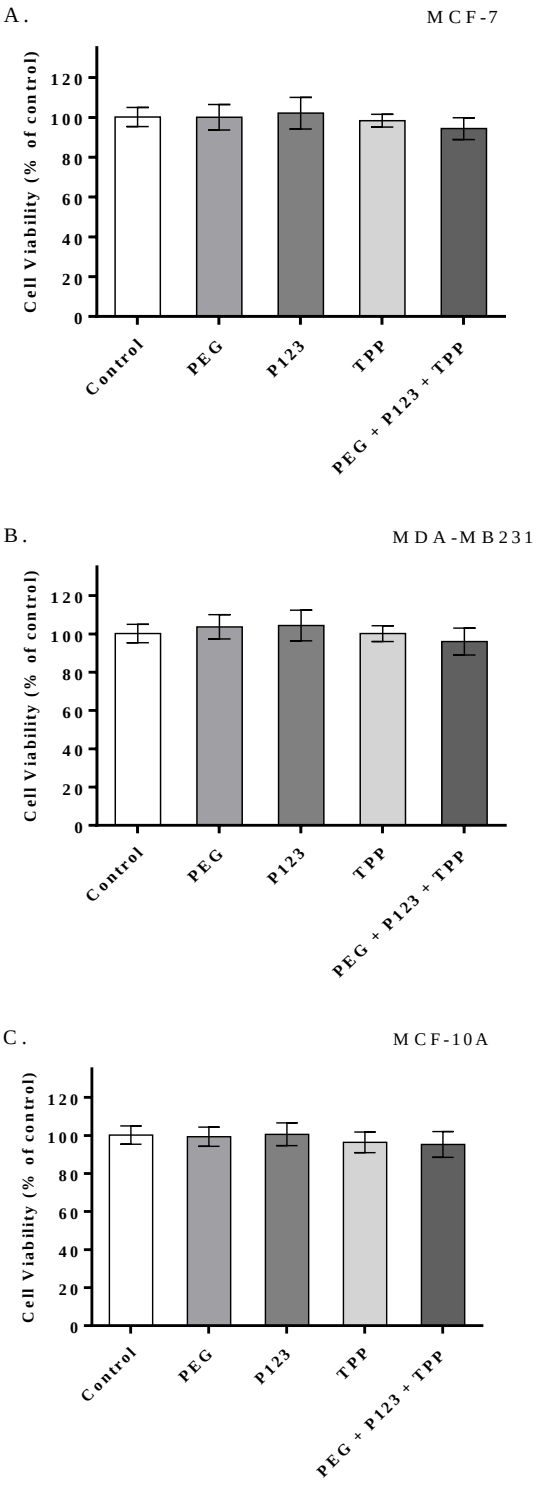
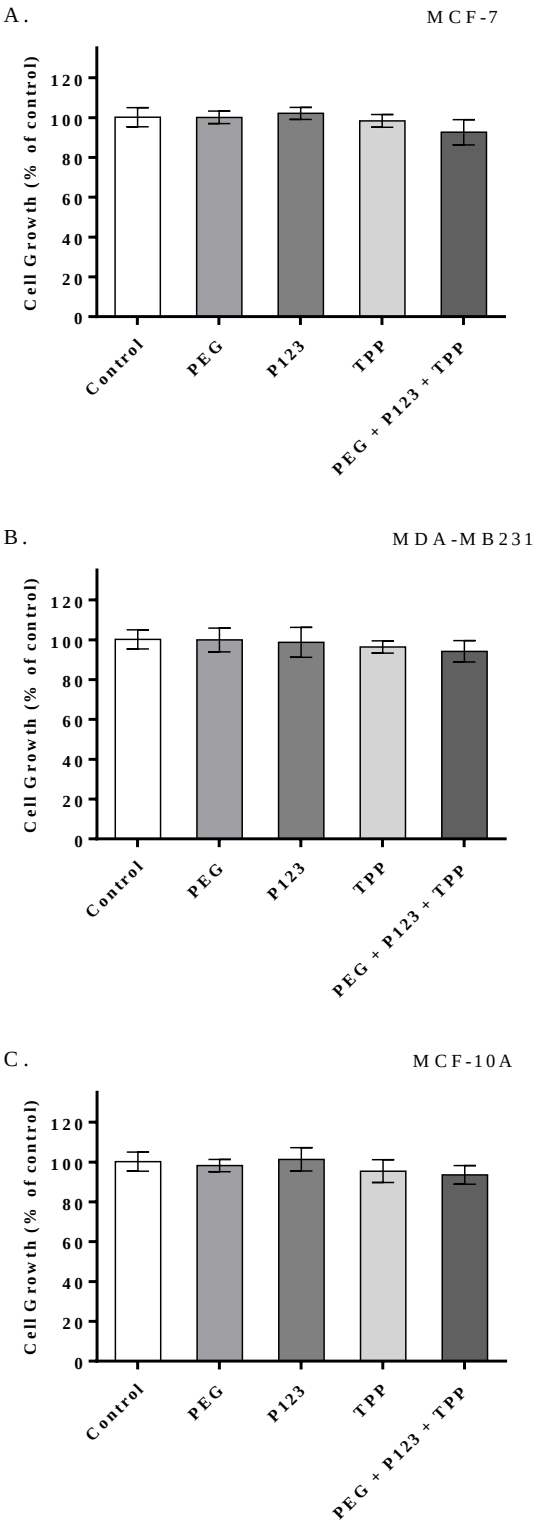


Figure 4.3.2S


A.
MCF-7
Cell Growth (% of control)
120
100
80
60
40
20
0
Control
PEG
P123
TPP
PEG + P123 + TPP
B.
MDA-MB231
Cell Growth (% of control)
120
100
80
60
40
20
0
Control
PEG
P123
TPP
PEG + P123 + TPP
C.
MCF-10A
Cell Growth (% of control)
120
100
80
60
40
20
0
Control
PEG
P123
TPP
PEG + P123 + TPP

CHAPTER 5

CONCLUSION

The purpose of this dissertation was to investigate the mitotoxic and cytotoxic effects of triphenylphosphonium cation (TPP) and *Origanum majorana* extract (OME) as well as PEGylated bioconjugate of OME with TPP (P-OME-TPP) against normal human breast epithelial and cancer cell lines. The novel concept of this dissertation was to use a bioconjugation approach that involves PEGylation of natural anti-tumor compounds and specific mitochondria targeting molecules for specifically preventing growth of breast cancer and possibly other types of cancers. Thus, its hypothesized that novel biodegradable and mitochondria-targeted bioconjugate of OME with PEG and TPP inhibits the growth of breast cancer cells.

Initially, the effect of estrogen (E2), TPP, and co-treatment of E2 with TPP in estrogen-dependent breast cancer (MCF-7), metastatic breast cancer (MDA-MB231), and normal epithelial breast (MCF-10A) cells was tested to determine possible mechanisms by which mitochondria contribute to the growth of breast cancer. Our findings revealed that E2-induced metabolic activity, proliferation, DNA damage, and ROS production were inhibited by TPP in MCF-7 cells. Since TPP is a mitochondria-targeted lipophilic cation and is shown to imbalance mitochondrial function, this inhibitory mechanism proposes for a potential contribution of mitochondria in estrogen-induced carcinogenesis. Overall, these findings offer a new perspective on the utility of mitochondria-targeted lipophilic cation TPP as a promising new class of targeted-drugs for the treatment of estrogen-dependent breast tumors.

Then, the potential cytotoxic effects of OME and P-OME-TPP in MCF-7, MDA-MB213, and MCF-10A cells were tested to determine the possible antitumor properties of OME and P-OME-TPP as a potential breast cancer therapy. Our findings demonstrated that 300 µg/mL of OME was able to significantly inhibit E2-induced metabolic activity and growth of MCF-7 cells. Furthermore, OME and P-OME-TPP were able induce cytotoxic effects in MCF-7 and MDA-MB231 cells, in time- and dose-dependent manner. Indeed, our findings revealed that synthesized P-OME-TPP was far more effective in exerting cytotoxic effects compared to OME in both MCF-7 and MDA-MB231 cells. Contrastingly, normal epithelial breast MCF-10A cells treated with OME and P-OME-TPP exhibited no change in regard to their viability and growth. Altogether, our findings offer a new perspective on the potential of *O. majorana* plant extract to be developed as a promising chemopreventive and new therapy against breast tumors.

5.1 Directions for future research

By providing a foundation for the utility of mitochondria-targeted cations, such as TPP, and natural antitumor compounds, such as OME, the results of this dissertation are intended to open the way for the rational design of mitochondria-targeted drugs that involve natural antitumor compounds directed against breast tumors and potentially many other tumor types.

Indeed, further investigations are needed to clarify the exact mechanisms by which E2-induced activities are inhibited by mitochondria-targeted cation. Since our results indicate that mitochondrial function involved in estrogen induced effects, further research regarding mitochondrial growth signaling and biogenesis are greatly needed. For instance,

investigation of mitochondrial signaling pathways that regulates progression of the cell cycle, which are involved in cell growth and proliferation, will clarify the E2-induced mechanisms that inhibited by mitochondria-targeted molecule.

Furthermore, investigating the exact mechanisms by which OME and P-OME-TPP are exhibiting cytotoxic effects are needed. First, chemical composition of *O. majorana* bioactive extract could be identified to characterize its active phenolic compounds. Also, several mechanisms, by which OME and P-OME-TPP is inducing its effects, could be investigated. For instance, the effects of OME and P-OME-TPP on cell cycle and apoptosis could be examined by measuring the levels of several cell cycle and apoptosis control proteins; such as cyclin-dependent kinase, survivin, tumor necrosis factor-α, caspase 8, caspase 3, PARP, and p21. Moreover, testing our hypothesis *in vivo* would be another area for future research. Xenografted tumors in chick embryos could be used to investigate OME and P-OME-TPP effect on tumor growth *in vivo*. Toxicity could be evaluated by comparing the number of deceased embryos in OME- P-OME-TPP-treated and control embryos. Finally, OME and P-OME-TPP ability to inhibit metastasis could be assessed by comparing the number of nodules in OME and P-OME-TPP treated tumors. This is a new direction of research that may provide the foundation for clinical research studies of mitochondria targeted and natural antitumor molecule-based therapy for the prevention and treatment of breast cancer.

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Garoub, M., Karbaschi, M., Roy, D., and Cooke, M. (2017). Influence of estrogen on mitochondria in estrogen-dependent breast cancer. (Prepared for submission).

Garoub, M., Karbaschi, M., Wnuk, S., and Cooke, M. (2017). Antitumor activity of extract and mitochondria-targeted bioconjugate of *origanum majorana* in breast cancer. (Prepared for submission).

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Roy, D., Morgan, M., Yoo, C., Deoraj, A., Roy, S., Yadav, V., Garoub, M., Assaggaf, H., and Doke, M. (2015). Integrated bioinformatics, environmental epidemiologic and genomic approaches to identify environmental and molecular links between endometriosis and breast cancer. *Int. J. Mol. Sci. 16*(10), 25285-25322.

Garoub, M., Roy, D., Das, J., and Wnuk, S. (2015). Influence of mitochondrial bioenergetics on the development of estrogen-dependent breast cancer. Poster presentation, AACR Annual Meeting, Philadelphia, PA.