Natural Product Berbamine Enhances the Efficacy of Doxorubicin Treatment for Triple Negative Breast Cancer

Jake Tyler

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NATURAL PRODUCT BERBAMINE ENHANCES THE EFFICACY OF DOXORUBICIN TREATMENT FOR TRIPLE NEGATIVE BREAST CANCER

by

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DEDICATION

To my wife, Jamie Tyler, my in-laws, Nancy and Jim Stuart, my sisters, and my parents.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Hexin Chen. Thank you for giving me the opportunity to work in your lab and for your efforts to improve my abilities as an independent researcher. Throughout my undergraduate and graduate education, Dr. Chen has instructed and mentored me to be a knowledgeable student, a capable researcher, and an active contributor to the scientific community. Thank you for helping me troubleshoot my projects and teaching me how to communicate my ideas effectively. These skills will benefit me tremendously in the future. I would also like to thank my committee members Dr. Marj Pena and Dr. Alan Waldman for their valuable feedback and guidance.
ABSTRACT

According to breast cancer statistics, 279,100 new cases are expected in the United States in 2020 (Siegel et al., 2020). Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer with a significantly shorter median overall survival compared to other subtypes of breast cancer. Unlike hormone-positive or HER2-positive breast cancers with effective hormonal or targeted therapies available, there has been very little clinical success from targeted therapies for TNBC. Doxorubicin (Dox) is one of the most used chemotherapy drugs. The only limitation of Dox treatment is its cytotoxicity. Berbamine dihydrochloride (BBM) is a natural benzylisoquinoline alkaloid that is extracted from the plant, Berbaris amurensis. It has been used in Ayurvedic and Chinese medicine to treat clinical patients with inflammation and cancer for many years. In order to improve the efficacy of Dox and reduce its toxicity, we screened a natural product called BBM that can synergistically inhibit cancer cell growth with Dox in TNBC. This study’s aim was to explore the therapeutic potential of the synergism of Dox with BBM. In this study, we identified that BBM has a synergistic effect with Dox and it increases Dox effectiveness by significantly decreasing the IC50 values of Dox in TNBC. We determined that BBM, an autophagy inhibitor, switches the death mode of Dox from autophagy to apoptosis. We also determined that BBM increases the extracellular ATP secretion and has the potential to trigger immunogenic cell death. These data strongly advocate for the therapeutic potential of the efficacy of Dox with BBM in triple negative breast cancer.
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<td>Apoptosis inducing factor</td>
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<td>ATG-12</td>
<td>Autophagy related protein12</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BBM</td>
<td>Berbamine dihydrochloride</td>
</tr>
<tr>
<td>BNIP3</td>
<td>BCL2 Interacting Protein 3</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</td>
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<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic initiation factor 2</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GRP78</td>
<td>Glucose regulated protein 78</td>
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<td>IC50</td>
<td>The half maximal inhibitory concentration</td>
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<tr>
<td>ICD</td>
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<td>IMDM</td>
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</tbody>
</table>
IRE1α ................................................................. Inositol requiring enzyme 1
LAMP-1 ............................................................. Lysosomal associated membrane protein 1
LDM ........................................................................ Low dose metronomic
MTD ........................................................................... Maximum tolerated doses
P2Y2 ......................................................................... Purinoceptor 2
PAC .............................................................................. Paclitaxel
PAS .............................................................................. Phagophore assembly site
PBS ............................................................................. Phosphate buffered saline
PERK ......................................................................... Protein kinase like endoplasmic reticulum kinase
PMA ........................................................................... Phorbol 12-myristate 13-acetate
RCF ............................................................................ Relative centrifugal force
SDS .............................................................................. Sodium dodecyl sulfate
TBST ........................................................................... Tris buffered saline-Tween 20
TNBC .......................................................................... Triple Negative Breast Cancer
UPR ............................................................................ Unfolded protein response
CHAPTER 1
INTRODUCTION

Triple Negative Breast Cancer

According to an estimation, approximately 1.38 million women get diagnosed with breast cancer yearly worldwide (Tomlinson et al., 2014). Breast cancer is characterized based on the absence or presence of three main receptors. These receptors are estrogen, progesterone, and HER-2. One of the most aggressive types of breast cancer is triple negative breast cancer (TNBC), which lacks these three main types of receptors. These three receptors usually fuel and provide nutrition to the non-TNBC breast cancers (Basu et al., 2008). Since the TNBC lacks the necessary receptors, treatments such as therapies and drugs that target estrogen, progesterone, HER-2 receptors cannot be used (Paradiso et al., 2017).

TNBC is the most aggressive subtype of breast cancer, with a significantly shorter median overall survival compared to other subtypes of breast cancer. Unlike hormone-positive or HER2-positive breast cancers with effective hormonal or targeted therapies available, there has been very little clinical success from targeted therapies for TNBC (Minami et al., 2011). Although TNBC has been assumed the potential protagonist of immunotherapy in breast cancer, the earlier studies showed that only 10% of patients responded to the therapy (Emens et al., 2019; Adams et al., 2019). The majority of TNBC patients still mainly depend on conventional chemotherapy. The two crucial concerns with the use of chemotherapy are high toxicity and drug resistance. Once the tumor is
resistance to standard chemotherapeutic drugs like anthracyclines or taxanes then only a few treatment options are available (Park et al., 2018).

Chemotherapy is typically designed to kill as many tumor cells as possible with the use of maximum tolerated doses (MTD) (Holohan et al., 2013). MTD treatment cannot be protracted in order to allow recovery of health tissues and to reduce toxicities. For aggressively growing tumors, during these therapeutic breaks, a burst in cell proliferation accompanied by manifestation of chemoresistance and accelerated angiogenesis are likely. There is an ongoing trend to move from MTD to the low dose metronomic (LDM) treatment to achieve optimal clinical outcomes (André et al., 2014; Pantziarka et al., 2016; Perroud et al., 2016; Scharovsky et al., 2009).

**Doxorubicin**

Doxorubicin (Dox) is one of the most used chemotherapy drugs. It is an anthracycline which was originally extracted from Streptomyces peucetius var. caesius in the 1970’s, and it is still used today in the treatment of several cancers such as breast, gastric, lung, myeloma, ovarian and thyroid (Thorn et al., 2011). Dox is known to do the intercalation into the Deoxyribonucleic acid (DNA) and therefore it disrupts the DNA repair by inhibiting an enzyme called topoisomerase II. The main function of the topoisomerase II is to unwind the DNA during transcription. Cancer cells need this enzyme in order to grow and replicate. Hence by inhibiting topoisomerase II, Dox stops the cancer progression by slowing down the cancer replication (Gewirtz et al., 1999).

Recent studies from animal models indicate that Dox, the most used chemotherapeutic drug for TNBC, administered in a chronic fashion induces apoptosis of
endothelial cells in the tumor microvasculature, therefore, inducing a prolonged antiangiogenic effect (Esser et al., 2001). In addition, Dox treatment of tumor cells can also induce signals of damage associated molecular pattern (DAMP) that can stimulate anti-tumor immunity. The only limitation of Dox treatment is its cytotoxicity (Apetoh et al., 2007; Casares et al., 2005; Garg et al., 2017; Ma et al., 2013; Poljaková et al., 2008). Dox is a cytotoxic drug. Cytotoxic drugs work by inhibiting the growth cycle and hence it effects the cells that grow rapidly (Poljaková et al., 2008). Like, cancer cells some normal cells also grow at a faster rate such as hair follicle and hence, in addition, to killing the cancer cells, Dox also kills the normal cells. Higher doses of Dox are extremely cytotoxic and produce a lot of side effects (Hosseinzadeh et al., 2019). Some common side effects of Dox are hair loss, nausea and vomiting, sores in the mouth, darkening of the soles, diarrhea, black stools, blood in the urine, pinpoint red spots on the skin, bruising, swelling of the feet, shortness of breath, and stomach pain. Majority of these side effects are dose dependent, and they get worse with an increase in doses. Because of all these side effects, long treatment of Dox with higher doses becomes very difficult (Buranrat et al., 2017; Hosseinzadeh et al., 2020).

**Paclitaxel**

Similar to Dox, paclitaxel (PAC) is another cytotoxic chemotherapy drug. It works by interfering the normal function of microtubules assembly, inhibiting the mitotic progression and killing the cancer cells by apoptosis (Jeong et al., 2016). Like Dox, PAC also has a lot of side effects such as low blood counts, hair loss, peripheral neuropathy, diarrhea, mouth sores, nausea, and vomiting. These side effects make it extremely hard to
prescribe PAC for a long period of time. PAC is currently used for breast cancer, cervical cancer, Kaposi sarcoma, pancreatic cancer, and lung cancer (Kassi et al., 2019).

Berbamine

Berbamine dihydrochloride (BBM), a natural benzylisoquinoline alkaloid is extracted from the plant, *Berberis amurensis*. It occurs as an active constituent in numerous medicinal plants and has an array of pharmacological properties. It has been used in Ayurvedic and Chinese medicine to treat clinical patients with inflammation and cancer for many years (Wang et al., 2009). BBM has been reported to have antitumor activities in various types of cancers, including myeloma, breast and lung cancers, especially targeting cancer stem cells (Gu et al., 2012; Hong et al., 2016; Liang et al., 2009; Yang et al., 2014). BBM has been found to inhibits autophagy by blocking the fusion between lysosome and autophagosome (Figure 1.2). Autophagy is a cellular recycling and self degradative process. The main role of autophagy is to degrade damaged organelle and misfolded proteins. Cancer cells sometimes uses autophagy as a tool for survival by getting rid of damaged organelles. BBM inhibits the autophagy and stops the cancer survival (Fu et al., 2018).

ER Stress

Recent studies have shown that Dox induces the endoplasmic reticulum stress and kills the cancer cells by autophagy, apoptosis and immunogenic cell death (Bagchi et al., 2018; Casares et al., 2005; Wang et al., 2004; Xiao et al., 2019). The endoplasmic reticulum (ER) is a cellular organelle which is found in all eukaryotic cells. The main
function of the ER to is to synthesize and fold the protein. The balance between the
protein synthesis and protein folding must be met for the ER to function properly (Hosoi
et al., 2015). The physiological stresses can increase the synthesis of the protein. As the
protein synthesis get increased it overwhelms the ER capacity to fold proteins. This over
production of proteins and disruption of the ER homeostasis results in the misfolding and
unfolding of the proteins (Xu et al., 2005). The accumulation of unfolded proteins
activates the unfolded protein response (UPR), which is a cellular stress response that
leads to the ER stress (Igwebuike et al., 2020). The UPR is carried out mainly by 2 ER
effector proteins. Protein kinase like endoplasmic reticulum kinase (PERK) and the
inositol requiring enzyme 1 (IRE1α). During the physiological state, in the absence of
UPR, PERK and IRE1α activation is suppressed by an ER chaperone called glucose
regulated protein 78 (GRP78). Under normal condition, both PERK and IRE1α are
bounded by GRP78, which inhibits their phosphorylation. During ER stress, GRP78
dissociates from PERK and IRE1α and binds to the unfolded proteins in order to refold
them back into their proper folding state (De la Cadena et al., 2013). In addition to the ER
stress, another protein called eukaryotic initiation factor 2 (eIF2α) plays an important role
in cell survival. The phosphorylation of the eIF2α at serine 51 is a well studied
mechanism which results in the inhibition of protein translation. The downregulation of
P-eIF2α reflects that the protein synthesis is increased which usually results in the
accumulation of misfolded proteins and hence causing the ER stress (Muaddi et al.,
2010). Both PERK and IRE1α are important markers of the ER stress while the P- eIF2α
indicates the attenuation of protein synthesis (Badiola et al., 2011).
Autophagy

Autophagy is a type of cell death in which the damaged cytosolic components are degraded by lysosomes. Autophagy plays an extremely significant role in cell survival (Glick et al., 2010). The macroautophagy (herein referred to as autophagy) is a highly conserved type of the autophagy in which a double membrane bound vesicles called autophagosomes engulfs the intracellular organelles and proteins. These autophagosomes are then delivered to lysosomes where they fuse with lysosomes for degradation (Kogel, 2015). The autophagy begins by the formation of the phagophore assembly site (PAS), the elongation of the PAS gives rise to the phagophore. A phagophore is a double membrane structure that encloses the damaged cytosolic components and misfolded proteins into a vesicle and this vesicle is called an autophagosome. These autophagosomes are then fused with the lysosomes for degradation (Figure 1.1). This fusion process is called autolysosome and it is the last stage of autophagy (Kaur et al., 2015).

In general, the autophagy is divided into three stages. The early stage, the middle stage, and the later stage. The early stage is the formation of phagophores. Beclin-1 is a protein which detects the expressions of these phagophore in this early stage of the autophagy. The middle stage is the formation of the autophagosomes. The autophagy related protein 12 (ATG-12) is a protein which detects the expressions of the autophagosomes in the middle stage of the autophagy. The later and the final stage is the fusion of the autophagosomes and lysosome where the degradation of the damaged organelles and misfolded proteins take places. LC3B-I detects the expressions of the autophagosomes in the later stage, and LC3B-II serves as an indicator of autolysosome of
autophagy (Kaur et al., 2015). During autophagy, non-lipidated soluble LC3B-I gets converted to phosphatidylethanolamine conjugated LC3B-II, which serves as an important and sensitive indicator of autophagy (Klionsky et al., 2016).

The main goal of the autophagy is to alleviate stress from the cells by removing the damaged organelles and misfolded proteins. Therefore, autophagy plays an important role in cell survival. Recent evidences have shown that autophagy can protect cancer cells from metabolic stresses because of its survival role (Alirezaei et al., 2010; Parzych et al., 2014; Zhang et al., 2009). Under normal conditions, autophagy occurs when SNAP29 interacts with STX17 and VAMP8, which drives autophagosome and lysosome fusion. BBM upregulates the expression of a protein called BCL-2 Interacting Protein 3 (BNIP3), which contains homology to BCL-2. BNIP3 can interact and inhibit the SNAP29 interaction with VAMP8, hence it inhibits the autophagy by blocking autophagosome and lysosome fusion (Figure 1.2). Autophagy helps to alleviate stress by the degradation of the damaged organelles and misfolded proteins. Recent evidence has shown that sometimes cancer cells use autophagy for cell survival. BBM inhibits the autophagy and hence stops the survival of cancer cells (Fu et al., 2018).

**Apoptosis**

Apoptosis is a type of programmed cell death. During apoptosis cells go through some morphological changes. Apoptotic cells start to shrink in size, which forces all the cell organelles to get tightly packed together (Figure 1.3). The cell membrane of these cells starts blebbing and eventually activates an essential executioner of apoptosis called caspase 3 (Elmore et al., 2007). During apoptosis, the executioner caspase 3 gets cleaved
by an initiator caspase (Caspase 2, Caspase 8, Caspase 9, or Caspase 10). The cleaved caspase 3 is an extremely important marker of apoptosis. During apoptosis, the cellular constituents of cells are not released into the surrounding by apoptotic cells. Apoptosis is a highly controlled type of programmed cell death, which does not lead to inflammation. (Porter et al., 1999).

There are two types of pathways of apoptosis, intrinsic and extrinsic pathways. In intrinsic pathways, cells sense the internal stress stimuli and respond to these signals by initiating apoptosis. These stress signals mostly include the DNA damage and biochemical stress signals. In extrinsic pathways, cells sense the external stress stimuli and responds to these external signals by initiating apoptosis (Fulda et al., 2006). Another type of apoptosis is called caspase 3 independent form of apoptosis. Recent studies have shown that a flavoprotein called apoptosis inducing factor (AIF), that is found in mitochondria, can induce apoptosis by a caspase 3 independent mechanism. Both the caspase 3 dependent and independent types of apoptosis have similar morphological features (Bai et al., 2015; Mansilla et al., 2005). Dox is known to disrupt the DNA repair through inhibiting an enzyme called topoisomerase II. The higher doses of Dox cause the DNA damage in cancer cells and activate the apoptosis through the intrinsic pathway. Most of the major therapies such as chemotherapy, immunotherapy and γ-irradiation kill the tumor by initiating apoptosis (Synowiec et al., 2015; Wang et al., 2004; Zhong et al., 2017).
**Immunogenic cell death**

Immunogenic cell death (ICD) is another type of cell death. The ICD is a specialized form of cell death that is characterized by the expression of damage associated molecular patterns (DAMPs) that are found in tumor microenvironments. The goal of ICD is to elicit an immune response. Some hallmarks of ICD are extracellular Adenosine triphosphate (ATP) release, exposure of calreticulin (CRT) on the cell surface and phagocytosis. The extracellular ATP release serves as find-me signals, to stimulate anti-cancer immunity (Garg et al., 2012; Zhou et al., 2019). The extracellular ATP is released by lysosomal exocytosis. This mechanism requires lysosomal associated membrane protein 1 (LAMP 1), which translocate to plasma membrane in a caspase 3 and pannexin 1 depended manner. The extracellular ATP release serves as find-me signal and macrophage detects this extracellular ATP signal by the help of a receptor called purinoceptor 2 (P2Y2) (Adamson et al., 2017; Martins et al., 2013; Radogna et al., 2018; Wang et al., 2013). The CRT is a chaperone that resides in the lumen of the ER. During ER stress, the phosphorylation of PERK and IRE1α leads to the translocation of CRT from the lumen of the ER to the surface of the cell. This translocation of CRT serves as eat-me signals for macrophages. The goal of the extracellular ATP release and translocation of CRT to the cell surface is for the macrophages to find these apoptotic cells and eat them. A recent study has shown that late stage autophagy inhibition enhances the CRT surface exposure (Li et al., 2016). The extracellular ATP release, translocation of CRT on the cell surface and phagocytosis serves as the important markers for ICD (Bezu et al., 2018; Feng et al., 2018; Garg et al., 2012; Keep et al., 2009; Obeid et al., 2006; Osman et al., 2017).
Hypothesis

The goals of this project were to see if the natural product BBM can be used in a synergistic manner with Dox to increase its efficacy. Dox is a cytotoxic chemotherapy drug and higher doses of Dox produces a lot of side effects (Hosseinzadeh et al., 2019). We wanted to see if BBM can enhance the effectiveness of Dox and if their combined therapy could be used to reduce Dox concentrations by decreasing its half maximal inhibitory concentration (IC50 value). The IC50 value represents the concentration of a drug that is required for it to reach the 50% inhibition of a specific biological function in vitro. In addition, we also wanted to find out if the synergism of BBM is just limited to Dox or if BBM could also has a synergistic effect with another chemotherapy drug called PAC and if its efficacy could also be increased.

The hypothesis of my research was that the natural product BBM can increase the efficacy of Dox by decreasing its IC50 values in TNBC. BBM can also increase the effectiveness of Dox by inhibiting the autophagy and switching the cell death mode to apoptosis. In addition, BBM also has the potential to induce ICD.
Figure 1.1: Overview of mammalian autophagy pathways. The macroautophagy initiates with the formation of the PAS. The elongation of the PAS gives rise to the phagophore. The phagophore expressions could be detected by Beclin-1. A phagophore is a double membrane structure that encloses the damaged cytosolic components and misfolded proteins into a vesicle and this vesicle is called an autophagosome. The autophagosome expressions could be detected by the ATG-12. This autophagosome are then fused with the lysosome for degradation. This fusion process is called autolysosome. LC3B serves as an indicator of autolysosome and hence it is a sensitive indicator for autophagy. This figure has been taken from (Kaur et al., 2015).
Figure 1.2: The proposed mechanism of BBM-mediated blockade of autophagosome and lysosome fusion. Under normal conditions, SNAP29 interacts with STX17 and VAMP8, which drives autophagosome and lysosome fusion. BBM upregulates the expression of BNIP3, which can interact and inhibit the SNAP29 interaction with VAMP8, hence it blocks the autophagosome and lysosome fusion. This figure has been taken from (Fu et al., 2018).
Figure 1.3: Morphology of apoptotic cell. It is a photomicrograph of a section of exocrine pancreas from B6C3F1 mouse. The arrows indicate apoptotic cells which are shrunk and condensed with cytoplasm. This figure has been taken from (Elmore et al., 2007).
CHAPTER 2
MATERIALS AND METHODS

Cell Culture and Reagents

Hs578T and MDA-MB-231 are human triple negative breast cancer cell lines. These cell lines were purchased from ATCC. Both cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (GE Healthcare Life Sciences), which contained 4 mM L-Glutamine, 4,500mg/L glucose and sodium pyruvate. In addition, 10% FBS and 1% of antibiotic antimycotic solution (10,000 ug/mL penicillin, 10,000 ug/mL streptomycin, and 25 ug/mL Amphotericin B) (Mediatech, Inc) were added to DMEM. Both cell lines were maintained at 37°C with 5% CO2 in a humidified incubator.

THP-1 is a human monocyte cell line. This cell line was obtained from Dr. Jun Zhu, University of South Carolina. This cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (GE Healthcare Life Sciences), which contained 4 mM L-Glutamine and HEPES. In addition, 10% FBS and 1% of antibiotic antimycotic solution (10,000 ug/mL penicillin, 10,000 ug/mL streptomycin, and 25 ug/mL Amphotericin B) (Mediatech, Inc) were added to IMDM. This cell line was also maintained at 37°C with 5% CO2 in a humidified incubator.

Dox (Cat number 25316-40-9) was purchased from Cayman Chemicals and it was dissolved in the Dimethyl sulfoxide (DMSO). PAC (Cat number 193532) was purchased from MP Biomedicals and it was dissolved in DMSO. BBM (Cat number 6078-17-7) was
purchased from Sigma-Aldrich and it was also dissolved in DMSO. The experiments were performed by treating the cells with Dox or BBM or Dox and BBM combined.

**MTT Assay**

MTT is an assay to examine cell viability. Hs578T and MDA-MB-231 cells were seeded at a density of 35,000 cells/well in triplicate in a 24 well plates. These cells were treated with Dox or BBM or Dox and BBM combined or PAC or PAC and BBM combined or DMSO for 24 hours. At the end of the incubation, media was removed, and MTT assay reagent called 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added in each well at the final concentration of 1mg/mL per well. These plates were incubated for 3 hours at 37°C with 5% CO2 in a humidified incubator. At the end of the incubation, media was removed, and the formazan precipitates were dissolved by adding 400 μl of DMSO in each well. OD values were measured at both 570 and 630 nm and final values were calculated by subtracting OD630 from OD570. The average values of each sample were used to plot the growth curve by using Microsoft Excel.

**Western Blot**

Hs578T and MDA-MB-231 cells were seeded at a density of 150,000 cells/well in a 12 well plate. These cells were treated with Dox or BBM or Dox and BBM combined or DMSO for 24 hours. At the end of the incubation, media was removed, and protein extracts were obtained from whole cell lysates. The protein was isolated by using cell lysis buffer, which contained Sodium orthovanadate, sodium fluoride, protease inhibitor
(Sigma), and mammalian protein extraction reagent (M-PER) (Thermofisher). This lysis buffer was used following the manufacturer’s instructions.

The protein quantification was performed by a Bradford assay. A mixture of 1 µL of protein and 9 µL of deionized water was added in a 96 well plate in triplicate. 10 µL of protein standards were added in the concentrations of 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, and 1 mg/mL. 150 µL of Coomassie protein assay reagent (Thermofisher) was added into each well of the 96 well plate. This plate was incubated in the dark for 10 minutes at room temperature. At the end of the incubation, a plate reader was used to read the results at 595 nm wavelength. A plot of protein standards was created in Microsoft Excel and protein concentrations of each sample was calculated based on the standards.

The proteins were denatured by adding the 6X Laemmli sample buffer and bringing its final concentration to 1X. All protein samples were boiled at 100°C for ten minutes for complete denaturation. The proteins samples and precision plus protein ladder (Bio-Rad) were loaded on a 12% acrylamide gels and these gels were loaded into the reservoir chamber (Bio-Rad). The reservoir chamber was filled with 1X protein running buffer (3 g Tris-HCl, 14.4 g glycine, 1 g SDS) and ran at 100 V for 2 hours at room temperature. Prior to transfer the gel, the transfer gel cassette (Bio-Rad) was assembled with two sponges, two pieces of filter paper, a piece of nitrocellulose paper (GE Healthcare Life Sciences). The transfer gel cassette was set up in the following order, from front to back: cassette, sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge, cassette. The cassette was closed and placed into the reservoir chamber along with an ice pack. The reservoir chamber was filled with 1X protein
transfer buffer (3 g Tris, 14.4 g glycine, and 10% methanol). The reservoir chamber was run at 230 mA for 1.5 hours at 4°C. After the gel transfer was complete the nitrocellulose membranes were removed and placed in the blocking buffer made of 5% milk (Nestle Carnation Instant Dry Milk, 20 g/L) mixed with Tris buffered saline-Tween 20 (TBST). These nitrocellulose membranes were incubated in the blocking buffer for 1 hour. At the end of the incubation, the nitrocellulose membranes were rinsed three times with TBST.

These nitrocellulose membranes were then probed with primary antibodies. All the primary antibodies were made in 5% bovine serum albumin (BSA) mixed with TBST in 1:1000 ratio (Table 2.1). The nitrocellulose membranes were incubated in primary antibodies overnight at 4°C on an orbital shaker. At the end of the incubation, these membranes were rinsed three times with TBST and then they were probed with HRP linked secondary antibody for 1 hour at room temperature on an orbital shaker. At the end of the incubation, these membranes were rinsed again three times with TBST. These membranes were finally removed from the TBST using tweezers and incubated in a 1:1 ratio mixture of either west pico plus chemiluminescent substrate (ThermoFisher Scientific) or west femto maximum sensitivity substrate (ThermoFisher Scientific) for 5 minutes. At the end of the incubation, these membranes were loaded on a cling wrap into the ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life) and the results were visualized according to optimal settings (Table 2.2). As the internal control for equal loading, a GAPDH (Santa Cruz Biotechnology) control was performed on each nitrocellulose membrane. Western blot images were quantified using GE ImageQuant Software.
ATP Quantification Assay

A Luminescent ATP Detection Assay Kit was purchased from Abcam (Cat no. ab113849). This kit was used to measure the extracellular ATP secretion. Hs578T and MDA-MB-231 cells were seeded at a density of 50,000 cells/well in 24 well plates. These cells were treated with Dox or BBM or Dox and BBM combined or DMSO for 24 hours. At the end of the incubation, media from each sample in the plates was moved into the microcentrifuge tubes. The media in these microcentrifuge tubes was gently mixed by pipetting up and down. A mixture of 50 µL of media and 25 µL of ATP substrate per sample was added together into the new microcentrifuge tubes. These tubes were incubated in the dark for 2 hours at room temperature. The ATP standards were also prepared in the concentrations of 0 µM, 0.0001 µM, 0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM according to the manufacture protocol. At the end of the incubation, media was removed and a luminometer machine was used to read the results. A plot of the ATP standards was created in Microsoft Excel and ATP concentration of each sample was calculated based on the standards.

Phagocytosis Assay

100,000 THP1 cells were added into ten 50 mm petri dishes. These cells were differentiated into macrophages for 3 days by adding 300nM of phorbol 12-myristate 13-acetate (PMA) per petri dish. These cells were maintained at 37°C with 5% CO2 in a humidified incubator. At the end of the incubation, macrophages were scraped off the 50 mm petri dishes.
Hs578T and MDA-MB-231 petri dishes were trypsinized by adding 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Mediatech) for 5 minutes in each petri dish. After the cells were trypsinized, they were neutralized with DMEM in 1:1 ratio. These cells were centrifuged at 500 (RCF) for 5 minutes at room temperature and then the cells were resuspended in new DMEM. These cells were counted with the help of a hemocytometer by adding trypan blue solution 0.4% (Thermo Fisher Scientific) into the cells. 5000,000 cells from each cell line were stained by adding 0.5 µL of cell tracker deep red dye (Life Technologies). These cells were incubated in the dark for 30 minutes. Both cell lines were seeded in a 1:3 ratio in 12 well plates (1 ratio of Hs578T or MDA-MB-231 and 3 ratios of macrophages), while keeping the total number of cells constant at 150,000 per well. After the cells were adhered to the surface of the plate overnight, they were treated with Dox or BBM or Dox and BBM combined or DMSO for 15 hours. These cells were maintained at 37°C with 5% CO2 in a humidified incubator.

At the end of the incubation, macrophages were co-cultured with Hs578T and MDA-MB-231 in a 1:3 ratio as previously described. These cells were also maintained at 37°C with 5% CO2 in a humidified incubator. At the end of the incubation, these cells were centrifuged at 500 RCF and then they were resuspended in 100 µL of 2% FBS. These cells were labeled with FITC anti-mouse/human CD11b antibody (BioLegend) at a concentration of 1:100 ratio and incubated for 1 hour on ice. At the end of the incubation, these cells were centrifuged at 500 RCF and then they were resuspended in 100 µL of 2% FBS. The fluorescence activated cell sorting (FACS) analysis was performed on these samples by using the flow cytometer. The results were analyzed based on cell tracker deep red eye and CD11b antibody. In FACS results, the CD11b (specific to
macrophages) was shown in FL1-A fluorescence channel and deep red dye (specific to Hs578T and MDA-MB-231) was shown in FL4-A fluorescence channel.

**Statistical Analysis**

The statistical analyses were conducted with Microsoft Excel. All data were shown as the mean ± standard deviation (SD). P-value of comparisons of two groups were analyzed using the two-tailed t-test. P-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ are indicated with (*), (**), and (***) respectively, and all considered statistically significant.
Table 2.1: The western blot antibodies

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<td>3398T</td>
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<tr>
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<td>7076S</td>
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**Table 2.1**: This table shows all the primary and secondary antibodies that were used in Western Blot. This table also show the concentration ratio, manufacturer and catalog number of all the antibodies.
Table 2.2: Optimal exposure settings for western blot detection

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<tr>
<td>Method</td>
<td>Chemilumination</td>
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</table>

Table 2.2: All samples were viewed using the ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences). The above settings were used for each western blot.
CHAPTER 3
RESULTS

Efficacy of Dox and PAC with BBM

The experiments were initiated by first screening the BBM to determine if BBM has any effect on cell growth of TNBC by itself. Hs578T cells were treated with BBM (5 \(\mu\text{M}, 10 \mu\text{M}, 20 \mu\text{M}, \text{and} 30 \mu\text{M}\)) for 24 hours. At the end of the incubation, MTT assay was performed to examine cells viability (Figure 3.1 A). We concluded that BBM by itself does not significantly inhibit the cell growth of Hs578T cells. The next steps were to screen some of the most common chemotherapy drugs; Dox and PAC along with BBM. We started the next experiment with Dox. Hs578T cells were treated with Dox (1 \(\mu\text{M}, 2.5 \mu\text{M}, 5 \mu\text{M}, \text{and} 10 \mu\text{M}\)) or BBM (5 \(\mu\text{M}, 10 \mu\text{M}, 20 \mu\text{M}, \text{and} 30 \mu\text{M}\)) or Dox and BBM combined or DMSO for 24 hours. Cell viability was conducted by MTT assay (Figure 3.1 B). The results showed that BBM synergistically increased the efficacy of Dox. Different doses of Dox and BMM was chosen to determine the best possible concentrations of this synergetic relationship between Dox and BBM. We noticed that BBM acted synergistically with Dox in a dose dependent manner as the cell viability values decreased with an increase in BBM concentration. The Dox 1\(\mu\text{M}\) average cell viability value without the BBM was 78 percent and the Dox 1\(\mu\text{M}\) and BBM 5 \(\mu\text{M}, 10 \mu\text{M}, 20 \mu\text{M}, \text{and} 30 \mu\text{M}\) combined average values were 69, 62, 56, 54 respectively. BBM 20 \(\mu\text{M}\) and 30 \(\mu\text{M}\) cells viability values were extremely close. This implied that BBM synergism might get less effective after the 20 \(\mu\text{M}\) concentration. In this experiment, the
results explained that the BBM 20 µM was the best concentration for the Dox and BBM synergistic relationship. The next sets of experiments were performed by keeping the BBM constant at 20 µM.

The next experiment was performed with PAC and BBM. Hs578T cells were treated with PAC (10 nM, 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM) or BBM (20 µM) or BBM and PAC combined or DMSO for 24 hours. At the end of the incubation, the cell viability was calculated by MTT assay (Figure 3.2 A). The results showed that BBM also has a synergistic relationship with PAC. Similar to Dox, BBM also acted synergistically with PAC in a dose dependent manner as the cell viability decreased with an increase in PAC concentration, in the presence of BBM. The BBM significantly decreased the PAC IC50 value. The IC50 value of PAC, without the BBM, was reached by PAC 50 nM, while the IC50 value with PAC and BBM combined was reached by PAC 25 nM (Figure 3.2 B). These results concluded that BBM increased the PAC efficacy by lowering its IC50 value.

Since the BBM concentration was now kept fixed at 20 µM, another series of experiment was performed with different concentrations of Dox and BBM. The goal of this experiment was to observe morphological changes of cells and to determine the best contractions of Dox and BBM for further studies. Hs578T cells were treated with Dox (1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM, 30 µM, 40 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours. At the end of the incubation, the cell viability was calculated by MTT assay (Figure 3.3 A). The results showed that BBM was more effective with Dox as compared to PAC. The BBM significantly decreased the IC50 value of Dox. The IC50 value with Dox without the BBM was reached by Dox 10 µM.
While IC50 value with Dox and BBM combined was reached by Dox 2.5 µM (Figure 3.3 B). We also noticed some morphological changes in cells treated with Dox without BBM as compared to the cells treated with Dox and BBM combined. The cells treated with Dox 1 µM without BBM appeared in clusters and looked inflamed while the cells that were treated with Dox 1 µM and BBM combined appeared in insolated patches and did not look inflamed. In Dox 5 µM without BBM, this clusters went away, and cells appeared more in insolated patches while the cells that were treated with Dox 5 µM and BBM combined appeared in very small insolated patches, and majority of cells appeared shrink with dense cytoplasm around them (Figure 3.4 A). In summary, these morphological changes suggested that BBM perhaps reduced the inflammation and changed the death mode of the cells. The morphology of cells that were treated with Dox 5 µM without the BBM looked similar to the cells that were treated with Dox 1 µM and BBM combined. These results indicated that perhaps the cells that were treated with Dox 5 µM without the BBM and that were treated with Dox 1 µM and BBM combined were dying from the same cell death pathway.

We decided to further pursue BBM experiments with Dox. The reason we chose Dox as compared to PAC was because recent studies showed that Dox might have more potential in therapy because it can induce ER stress and kill cancer cells by autophagy, apoptosis and ICD (Bagchi et al., 2018; Casares et al., 2005; Wang et al., 2004; Xiao et al., 2019). In addition, the IC50 values from the results indicated that BBM was more effective with Dox as compared to PAC.

To further study the relation of Dox and BBM we chose another TNBC cell line called MDA-MB-231 to perform the MTT assay. The MDA-MB-231 cells were treated
with Dox (1 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM) or BBM (20 μM) or Dox and BBM combined or DMSO for 24 hours. At the end of the incubation, the cell viability was again calculated by MTT assay (Figure 3.3 C). BBM was also very effective with Dox in MDA-MB-231 cell line as it significantly decreased the IC50 value. The IC50 value with Dox without the BBM was reached by Dox 5 μM. While IC50 value with Dox and BBM combined was reached by Dox 2.5 μM (Figure 3.3 D). The morphology of MDA-MB-231 cells looked different as compared to the morphology of Hs578T cells. The cells treated with Dox 1 μM without the BBM did not looked inflamed and they looked similar to the cells that were treated with Dox 1 μM and BBM combined. In Dox 5 μM without the BBM, these cells also looked similar to the cells that were treated with Dox 5 μM and BBM combined. The majority of cell treated Dox 5 μM and BBM combined, appeared to shrink with dense cytoplasm around them (Figure 3.4 B). In summary, these different morphological changes suggested that BBM cell death pathways were perhaps different in Hs578T and MDA-MB-231.

Effects of Dox with BBM on ER Stress

The next experiments were designed to study the ER stress, autophagy and apoptosis related proteins. Both Hs578T and MDA-MB-231 cells were treated with Dox (1 μM, 2.5 μM, 5 μM, 10 μM) or BBM (20 μM) or Dox and BBM combined or DMSO for 24 hours. Western blot analysis was used at the end of the treatment to study the protein expressions of both cell lines. The two main ER stress proteins are PERK and IRE1α. Under normal condition, both PERK and IRE1α are bounded by GRP78 which inhibits their phosphorylation. During ER stress, GRP78 dissociates from PERK and
IRE1α and binds to the unfolded proteins in order to refold them back into their proper folding state (De la Cadena et al., 2013). In addition to the ER stress another protein called P-eIF2α plays an important role in cell survival. Phosphorylation of the eIF2α at serine 51 is a well studied mechanism which results in the inhibition of protein translation. The downregulation of P-eIF2α reflects that the protein synthesis is increased which usually results in the accumulation of misfolded proteins and hence causing the ER stress (Muaddi et al., 2010).

The Hs578T western blot results showed that both PERK and IRE1α were downregulated in Dox with BBM combined doses. The expressions of both the PERK and IRE1α were observed at its peak in Dox 1 µM and BBM combined dose. The P-eIF2α expressions showed that the protein translation was inhibited in both the lower and higher Dox with BBM combined doses. In summary, these results indicated that the ER stress was at its peak in lower concentration while in higher concentrations of Dox with BBM combined, the ER stress was going down. The P-eIF2α expressions indicated that the protein translation was inhibited at both lower and higher concentration of Dox with BBM combined doses (Figure 3.5 A). The MDA-MB-231 western blot results showed that both PERK and IRE1α were also downregulated in Dox with BBM combined doses. The expressions of both the PERK and IRE1α in MDA-MB-231 was different as compared to the Hs578T. In MDA-MB-231, the ER stress proteins expressions were not at its peak in Dox 1 µM and BBM combined dose as previously observed in Hs578T. The P-eIF2α expression was downregulated in Dox 1 µM and BBM combined dose and it was upregulated in the higher doses of Dox with BBM combined (Figure 3.5 B). In summary, these results showed that ER stress was going down in both lower and higher doses of
Dox with BBM combined. The protein synthesis was accelerated in Dox 1 μM and BBM combined dose, but it was inhibited in higher concentrations of Dox with BBM combined.

**Effects of Dox with BBM on Autophagy**

Autophagy is divided into three stages. The early stage, the middle stage, and the later stage. The early stage is the formation of phagophores. The Beclin-1 is a protein which detects the expressions of these phagophore in this early stage of the autophagy. The middle stage is the formation of the autophagosomes. The ATG-12 is a protein which detects the expressions of the autophagosomes in the middle stage of the autophagy. The later and the final stage is the fusion of the autophagosomes and lysosome where the degradation of the damaged organelles and misfolded proteins take places. LC3B-I detects the expressions of the autophagosomes in the later stage, and LC3B-II serves as an indicator of autolysosome of autophagy. Thus, LC3B-II serves as a sensitive indicator of autophagy (Kaur et al., 2015).

Hs578T results indicated that Beclin-1 expressions went down in the dose dependent manner, the lower doses of Dox with BBM combined has higher expressions of Beclin-1 as compared to the higher doses of Dox with BBM combined. These expressions explained that phagophore formation was increased in lower doses of Dox with BBM combined as compared to the higher doses of Dox with BBM combined. The ATG-12 expressions were not significantly changed in lower doses of Dox with BBM combined, but they were decreased slightly in higher doses of Dox with BBM combined. These results indicated that the autophagosomes formation went down in the higher dose
of Dox with BBM while at the lower dose of Dox with BMM, there was no significant change in the autophagosomes formation. The final stage of the autophagy was studied by the conversion of the LC3B-I to LC3B-II, which is the fusion of the autophagosomes and lysosome that leads to degradation. Because of the degradation, down regulation of LC3B-II expressions serves as an indicator that cell died by the autophagy. No significant expressions of LC3B-II were observed in Dox concentrations without the BBM, indicating that Dox without the BBM was killing the cancer cells by autophagy. In Dox with BBM combined, the LC3B-II expression was at peak in Dox 1 µM and BBM combined, and its expressions started to downregulate in higher doses of Dox with BBM combined. These results indicated that the lower doses of Dox with BBM combined inhibited the autophagy, while at the higher doses of Dox with BBM combined, the inhibition of autophagy got reversed and cell started to die by autophagy (Figure 3.5 A).

In summary, in the lower doses of Dox with BBM combined, the phagophores and autophagosomes expressions were not significantly changed. The highest peak of LC3B-II was observed in Dox 1 µM with BBM combined, this peak indicated that the autophagy was inhibited. In the higher doses of Dox with BBM combined, the phagophore and autophagosomes expressions were decreased. The LC3B-II signals were downregulated indicating that inhibition of autophagy was reversed, and cell were dying by autophagy.

The MDA-MB-231 results indicated that there was not a significant change in the expressions of Beclin-1 and ATG-12 in the cells that were treated with Dox with BBM combined, indicating that phagophore and autophagosomes expressions were not significantly changed. The LC3B-II expressions were observed in all the Dox with BBM
combined doses, indicating that the autophagy was entirely blocked throughout the treatment. In summary, both the lower and higher doses of the Dox with BBM combined had similar effects. The phagophores and autophagosomes expressions were not significantly changed and the autophagy was entirely inhibited (Figure 3.5 B).

Effects of Dox with BBM on Apoptosis

During apoptosis, the executioner caspase 3 gets cleaved by an initiator caspase (Caspase 2, Caspase 8, Caspase 9, or Caspase 10). The cleaved caspase 3 is an extremely important marker of apoptosis. Another type of apoptosis is called caspase 3 independent form of apoptosis. Recent studies have shown that a flavoprotein called AIF, that is found in mitochondria, can induce apoptosis by a caspase 3 independent mechanism. Both the caspase 3 dependent and independent types of apoptosis have similar morphological features (Bai et al., 2015; Mansilla et al., 2005).

Hs578T results showed that in Dox without the BBM treatment, cleaved caspase-3 expressions only appeared in Dox 10 µM, while in cells treated by Dox with BBM combined, low expressions of cleaved caspase 3 started to appear in Dox 1µM with BBM combined and these expressions were upregulated in a dose dependent manner. The Dox 5 µM with BBM combined, showed very significant upregulation of the cleaved caspase 3 expressions, indicating that the addition of BBM with Dox treatment changes the Dox cell death mode from autophagy to apoptosis. These results also indicated that the BBM started to change the cell death mode and initiated the apoptosis in both the lower and higher doses of the Dox (Figure 3.5 A). The MDA-MB-231 results showed that that the cleaved caspase 3 expression was at peak at Dox 1 µM and BBM combined, and its
expressions started to downregulate in higher doses of Dox with BBM combined. These results indicated that the lower doses of Dox with BBM combined triggered the caspase 3 dependent apoptosis while higher doses of Dox with BBM combined, perhaps triggered the apoptosis through caspase 3 independent pathway (Figure 3.5 B). The GAPDH was used as the loading control for the western blot and all the protein expressions were normalized based on the GAPDH expressions.

In summary, the Hs578T results showed that the both lower and higher doses of Dox with BBM combined triggered the caspase 3 dependent apoptosis. The MDA-MB-231 results showed that lower doses of Dox with BBM combined triggered the caspase 3 dependent apoptosis while higher doses of Dox with BBM combined perhaps triggered the caspase 3 independent apoptosis.

Effects of Dox with BBM on ICD

The goal of the ATP quantification experiments was to determine if the BBM had the potential to trigger ICD by increasing the extracellular ATP secretion. ICD is a specialized form of cell death that is characterized by the expression of the DAMPs that are found in tumor microenvironments. The goal of ICD is to elicit an immune response. Some hallmarks of ICD are extracellular ATP release, exposure of CRT on the cell surface and phagocytosis. The extracellular ATP release serves as find-me signals, to stimulate anti-cancer immunity. The extracellular ATP is released by lysosomal exocytosis. This mechanism requires lysosomal associated membrane protein 1 (LAMP 1), which translocate to plasma membrane in a caspase 3 and pannexin 1 depended manner (Garg et al., 2012; Zhou et al., 2019).
Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours. At the end of the incubation, the extracellular ATP quantification was measured. Hs578T results indicated that the lower doses of Dox with BBM combined did not significantly triggered the secretion of the extracellular ATP while the higher doses of the Dox with BBM combined triggered the secretion of the extracellular ATP. The extracellular ATP secretion started to increase at Dox 5 µM and BBM combined. The cleaved caspase 3 is required along with the autophagy triggered the secretion of the ATP (Figure 3.6 A). At Dox 5 µM and BBM combined, cleaved caspase 3 expressions were upregulated, and autophagy was also observed, hence all the conditions were met for the secretion of the extracellular ATP. In summary, the lower doses of Dox with BBM combined did not trigger the secretion of the extracellular ATP while the higher doses of Dox with BBM combined triggered the secretion of the extracellular ATP.

The MDA-MB-231 results showed that the extracellular ATP secretion was not significantly triggered by the Dox with BBM combined doses (Figure 3.6 B). This ATP pattern was not proportional to the cleaved caspase 3 pattern of Dox 1 µM and BBM combined. In addition to the cleaved caspase 3 the secretion of the extracellular ATP also requires the autophagy. In Dox 1 µM and BBM combined, the western blot results indicated that the autophagy was entirely inhibited throughout the treatment. Hence, these results showed that the extracellular ATP was not secreted because both conditions of the ATP secretion were not met. In addition to the inhibition of autophagy, the higher doses of Dox with BBM combined did not also trigger the secretion of the extracellular ATP because the cell perhaps died through caspase 3 independent form of apoptosis.
The phagocytosis assay was performed to determine if the macrophages could track the secretion of ATP from the apoptotic cells. The extracellular ATP release serves as find-me signal and macrophage detects this extracellular ATP signal by the help of a P2Y2 receptor (Adamson et al., 2017; Martins et al., 2013; Radogna et al., 2018; Wang et al., 2013). CRT is a chaperone that resides in the lumen of the ER. During ER stress, the phosphorylation of PERK and IRE1α leads to the translocation of CRT from the lumen of the ER to the surface of the cell. This translocation of CRT serves as eat-me signals for macrophages. The goal of the extracellular ATP release and translocation of CRT to the cell surface is for the macrophages to find these apoptotic cells and eat them. A recent study has shown that late stage autophagy inhibition enhances the CRT surface exposure (Li et al., 2016). The extracellular ATP release, translocation of CRT on the cell surface and phagocytosis serves as the markers for the ICD.

The Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 5 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 15 hours and co-culture with macrophages for 3 hours. At the end of the incubation, the phagocytosis assay was performed by FACS. The results were analyzed by staining both Hs578T and MDA-MB-231 cells by the cell tracker deep red dye and macrophages were labeled with FITC anti-mouse/human CD11b. In FACS, the CD11b (specific to macrophages) was shown in FL1-A fluorescence channel and deep red dye (specific to Hs578T and MDA-MB-231) was shown in FL4-A fluorescence channel.

The Hs578T results showed that the number of macrophages eating the apoptotic cells was increased in dose dependent manner. The Dox 5 µM and BBM combined had the highest peak. This peak was proportional to the extracellular ATP secretion. The
phagocytosis was also observed in Dox 1 µM and BBM combined (Figure 3.7 A and B). This phagocytosis expression was not proportional with the extracellular ATP secretion, but the western blot results showed that in Dox 1 µM and BBM combined both ER stress proteins PERK and IRE1α were phosphorylated and the later stage autophagy was blocked. All these results indicated that the Dox 1 µM and BBM combined phagocytosis was perhaps led by CRT exposure to the cell surface. The MDA-MB-231 results did not show any significant phagocytosis (Figure 3.7 C and D). These results were proportional with the extracellular ATP release and the western blots results did not show the phosphorylation of ER stress proteins either.
Figure 3.1: MTT of Hs578T cells with Dox and different BBM concentrations treatment. Hs578T cells were treated with BBM (5 µM, 10 µM, 20 µM, 30 µM) (A) and Dox (1 µM, 2.5 µM, 5 µM, 10 µM) or Dox and BBM combined or DMSO for 24 hours (B). At the end of the treatment, MTT assay reagent called 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to examine the cells viability. P-values of p < 0.05, p < 0.01, and p < 0.001 are indicated with (*), (**), and (***), respectively, and all considered statistically significant.
Figure 3.2: MTT of Hs578T cells with PAC and BBM treatment. Hs578T cells were treated with PAC (10 nM, 25 nM, 50 nM, 100 nM, 150 nM, 200 nM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours (A and B). At the end of the treatment, MTT assay reagent called 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to examine the cells viability. P-values of p < 0.05, p < 0.01, and p < 0.001 are indicated with (*), (**), and (***) respectively, and all considered statistically significant.
MTT of Hs578T with Dox and BBM Treatment

A

MTT of Hs578T with Dox and BBM Treatment

B
Figure 3.3: MTT of Hs578T and MDA-MB-231 cells with Dox and BBM treatment. Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM, 30 µM, 40 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours (A, B, C and D). At the end of the treatment, MTT assay reagent called 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to examine the cells viability. P-values of p < 0.05, p < 0.01, and p < 0.001 are indicated with (*), (**), and (***) respectively, and all considered statistically significant.
Morphological changes of Hs578T with Dox and BBM Treatment

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Dox 1 and BBM: ![Image](image5.png)

Dox 5 and BBM: ![Image](image6.png)
Morphological changes of MDA-MB-231 with Dox and BBM Treatment

Figure 3.4: Morphological changes of Hs578T and MDA-MB-231 cells with Dox and BBM treatment. Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 5 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours (A and B). These images were captured by a compound microscope at the end of the treatment.
Western Blot of Hs578T with Dox and BBM Treatment

- **P-eIF2α**
- **PERK**
- **IRE1α**

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Figure 3.5: Western Blot of Hs578T and MDA-MB-231 cells with Dox and BBM treatment. Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 2.5 µM, 5 µM, 10 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours. Western blot analysis was used at the end of the treatment to show the protein expressions of ER stress, autophagy, and apoptosis related proteins (A and B). P-eIF2α, PERK, and IRE1α are ER stress proteins. Beclin-1, ATG-12, and LC3B 1 and 2 are autophagy proteins. Cleaved caspase 3 is an apoptosis protein. GAPDH was used as a loading control.
Figure 3.6: The extracellular ATP quantification of Hs578T and MDA-MB-231 cells with Dox and BBM treatment. Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 24 hours. At the end of the treatment, extracellular ATP quantification was measured (A and B). P-values of p < 0.05, p < 0.01, and p < 0.001 are indicated with (*), (**), and (***) respectively, and all considered statistically significant.
Phagocytosis Assay of Hs578T with Dox and BBM Treatment

A

B

Phagocytosis Assay of Hs578T with Dox and BBM Treatment

Fold Change

Control | Dox 1 | Dox 5

Dox (µM) | BBM (µM)
Figure 3.7: Phagocytosis assay of Hs578T and MDA-MB-231 cells with Dox and BBM treatment. Hs578T and MDA-MB-231 cells were treated with Dox (1 µM, 5 µM) or BBM (20 µM) or Dox and BBM combined or DMSO for 15 hours and co-cultures with macrophages for 3 hours. At the end of the incubation, the phagocytosis assay was performed by doing FACS (A, B, C, and D). P-values of p < 0.05, p < 0.01, and p < 0.001 are indicated with (*), (**), and (***), respectively, and all considered statistically significant.
CHAPTER 4

CONCLUSION

Efficacy of Dox and PAC with BBM

The goal of this study was to determine if BBM can enhance the efficacy of chemotherapy drugs. The first step was to screen chemotherapy drugs and we started by screening some of the most common chemotherapy drugs; Dox and PAC. These chemotherapy drugs were chosen because of their ability to inhibit the cell growth and induce apoptosis. The Hs578T and MDA-MB-231 cell lines were chosen for the experiments because they are aggressive TNBC cell lines.

The efficacy of BBM was determined by MTT assays. The BBM concentration was fixed at 20 µM because of its effectiveness with Dox. The BBM synergism was more effective in Dox as compared to the PAC because Dox with BBM combined IC50 value was significantly lower as compared to the PAC with BBM combined IC50 value. The efficacy of Dox was increased by the BBM in both the TNBC cell lines; Hs578T and MDA-MB-231. The morphological changes were different in Hs578T as compared to the MDA-MB-231, indicating that the cells were perhaps dying of different cell death pathways.

Effects of Dox with BBM on ER Stress, Autophagy, and Apoptosis

The ER stress, autophagy and apoptosis effects were determined by western blot. The western blot results of Hs578T showed that the ER stress was caused in the lower
doses of Dox with BBM combined, because of the inhibition of the autophagy. The autophagy was responsible to degrade the misfolded proteins and since the autophagy was inhibited the misfolded proteins initiated the UPR and hence causing the ER stress. The ER stress went away in the higher doses of Dox with BBM combined because the autophagy inhibition was reversed, and cells were dying by both the autophagy and apoptosis. The results of the treatment of Dox without the BBM showed that the majority of the cells were only dying from the autophagy while the addition of BBM showed that in the lower doses the cell death mode was switched from autophagy to caspase 3 dependent apoptosis and in higher doses the cells were dying by both the autophagy and the caspase 3 dependent apoptosis. The western blot results of MDA-MB-231 showed that the ER stress expressions were not significantly increased in the lower doses of Dox with BBM combined. These results explained that even when the autophagy was inhibited the misfolded proteins were not accumulated because the cells were dying by apoptosis. The LC3B-II expressions explained that the autophagy was inhibited throughout the entire treatment of Dox with BBM combined. The cleaved caspase 3 expressions were downregulated in a dose dependent manner. These results indicated that the lower doses of Dox with BBM combined triggered the caspase 3 dependent apoptosis while higher doses of Dox with BBM combined, perhaps triggered the caspase 3 independent apoptosis.

**Effects of Dox with BBM on ICD**

The extracellular ATP secretion and phagocytosis are the important hallmarks of ICD. The results showed that the Hs578T extracellular ATP pattern was different as
compared to the MDA-MB-231. In Hs578T, the lower doses of Dox with BBM combined did not trigger the extracellular ATP secretion while the higher doses of Dox with BBM combined triggered the secretion of the extracellular ATP. The Hs578T phagocytosis results showed that the lower doses of Dox with BBM combined perhaps led the phagocytosis by the CRT exposure to cell surface caused by the ER stress and late stage autophagy inhibition. The higher doses of Dox with BBM combined led the phagocytosis by the secretion of the extracellular ATP. In MDA-MB-231, the extracellular ATP secretion was not significantly increased by both the lower and higher doses of Dox with BBM combined. This is because both the cleaved caspase 3 and autophagy are required for the secretion of the extracellular ATP. The autophagy was inhibited throughout the BBM treatment and hence it did not increase the extracellular ATP secretion significantly. The MDA-MB-231 results also did not show any significant phagocytosis activity. These results were proportional with the extracellular ATP secretion and ER stress led CRT cell surface exposure because neither the extracellular ATP secretion nor the ER stress was significantly observed by Dox with BBM combined treatment.

Conclusion

In conclusion, this study’s aim was to explore the therapeutic potential of the synergism of Dox with BBM. The MTT assay results showed that BBM has a synergistic effect with Dox and it increases Dox efficacy by significantly decreasing the IC50 values of Dox in both Hs578T and MDA-MB-231 cell lines. Because of BBM’s ability of decreasing the IC50 value of Dox, the combinational treatment of Dox with
BBM could be used to decrease the Dox concentration without compromising its effectiveness. The decrease in the Dox concentration would also decrease its cytotoxicity and side effects. The western blot results showed that BBM, an autophagy inhibitor, switches the cell death mode of Dox from autophagy to apoptosis. Recent studies have shown that certain breast cancers become resistant to Dox chemotherapy (Han et al., 2019; Lovitt et al., 2018). The combined treatment of Dox with BBM could also be used to switch the cell death mode from autophagy to apoptosis and hence it perhaps could be used to treat the chemoresistance of certain breast cancers. In Hs578T, the extracellular ATP results showed that BBM increases the extracellular ATP secretion. The phagocytosis assay showed that the extracellular ATP secretion served as find-me signals for macrophages and it has the potential to trigger ICD. In Hs578T, the combined treatment of Dox with BBM killed the cancer cells by three different types of cell deaths: autophagy, apoptosis and ICD. The MDA-MB-231 cells perhaps died by two different forms of apoptosis: caspase 3 dependent and caspase 3 independent apoptosis.

The BBM future studies could be done on finding the specific cell death pathways and trying to understand the role of BBM in switching the cell death mode from autophagy to apoptosis in Dox. Further chemotherapy drugs could also be studied to figure out if BBM could also increase the efficacy of more chemotherapy drugs. In addition, in vivo studies could be done to study the synergistic effects of BBM with Dox and its ICD potential. These data strongly advocate for the therapeutic potential of the efficacy of BBM with Dox in triple negative breast cancer.
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