Repositioning Propranolol to Block Mitogenic Signaling in Breast Cancer

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REPOSITIONING PROPRANOLOL TO BLOCK MITOGENIC SIGNALING IN BREAST CANCER

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Doctoral Program in Biosciences

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Dedication

To my sister, who has always been by my side since the day I can remember. I wouldn’t have made it through my graduate career without you. I have looked up to you for so many years and have always been amazed by everything you do. You are my rock. Thank you. I love you.
REPOSITIONING PROPRANOLOL TO BLOCK MITOGENIC

SIGNALLING IN BREAST CANCER

by

Alexa Montoya B.S

DISSERTATION

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Abstract

Breast cancer is the second most diagnosed cancer among women and is about 30% of all new cases of female cancers each year. It is projected that 1 in 8 every U.S. woman (about 13%) develop invasive breast cancer over the course of her lifetime. While advances in cancer research have made it possible to elucidate several breast cancer genomic subtypes, and develop new novel therapies, many of these agents are associated with significant toxicity, as well as high costs. A retrospective cross-sectional study of 404 breast cancer patients was performed to determine the effect of β-blocker usage on tumor proliferation. Our analysis revealed that non-selective β-blockers, but not selective β-blockers, reduced tumor proliferation by 66% (p < 0.0001) in early-stage breast cancer compared to non-users. Substantial evidence supports the use of inexpensive β-AR antagonists against a variety of cancers. Propranolol is a β-AR antagonist (β-Adrenergic Receptor blockers) that has been effectively treating coronary heart disease and high blood pressure since the 1960’s. Prospective and retrospective data published by our lab and others suggest that non-selective β-AR antagonists are effective at reducing proliferative rates in breast cancers, however the mechanism by which this occurs is largely unknown.

The goal of this project was to better understand the molecular pathway and identify molecular markers affected when repurposed drugs such as propranolol are administered to patients with early stage and late-stage breast cancer. The anti-proliferative effects of Propranolol as well as PND was measured in a panel of breast cancer lines cancer cells, which include the highly aggressive MDA-MB-231, MDA-MB-468, MCF-7, and HCC70 cells, to evaluate synergistic activity, but the results indicated no synergistic activity was found and in one cell line showed antagonistic effects.
# Table of Contents

Abstract.................................................................................................................................................. v

Table of Contents ................................................................................................................................... vi

List of Tables ........................................................................................................................................ viii

List of Figures ......................................................................................................................................... ix

Chapter 1: Use of non-selective β-blockers is associated with decreased tumor proliferative indices in early stage breast cancer ......................................................................................................... 1
  
  Introduction ......................................................................................................................................... 1
  
  β1-AR and β3-AR are overexpressed in breast cancer ........................................................................... 2
  
  Use of non-selective β-blockers is associated with reduced tumor proliferation in early stage breast cancer patients ......................................................................................................................... 4

  Popranolol reduced the breast cancer proliferative index in a window of opportunity case study ..................................................................................................................................................... 4

  β-blockade reduces the proliferative index of breast cancer cell lines ................................................. 5

  Discussion ............................................................................................................................................ 6

  Materials and Methods ....................................................................................................................... 10

  Tables .................................................................................................................................................. 15

  Figures ............................................................................................................................................... 17

Chapter 2: The Beta-Adrenergic Receptor Antagonist Propranolol Alters Mitogenic and Apoptotic Signaling in Late Stage Breast Cancer ..................................................................................... 22
  
  Introduction ........................................................................................................................................ 22

  Neoadjuvant Treatment of a Late Stage Breast Cancer Patient with Propranolol ......................... 23

  Propranolol Alters Breast Cancer Cell Cycle Progression ............................................................... 23

  Propranolol Enhances p53 Activation in Breast Cancer Cells ......................................................... 25

  Propranolol Induces Apoptosis in Breast Cancer Cells ................................................................. 25

  Discussion .......................................................................................................................................... 26

  Materials and Methods ...................................................................................................................... 28

  Figures .............................................................................................................................................. 32

Chapter 3: Identifying Potential Synergistic Effects of Propranolol and PND on Breast Cancer Cell Lines .................................................................................................................................................. 36
  
  Introduction ...................................................................................................................................... 36
Chapter 4: Testing Additional Breast Cancer Cell Lines HCC70 and MDA-MB-468 to try to Better Understand the Variability of Effects of Propranolol and PND Treatment

Introduction
Propranolol and PND prove to be Ineffective on the Normal Breast Epithelial MCF10-A Cell Line
Propranolol and PND Induce Possible Apoptotic Activity in the HCC70 Cell Line
Propranolol and PND Work Together to Reduce Cell Proliferation of MDA-MB-468 Cells

Discussion
Materials and Methods
Tables
Figures
Conclusion
References
Vita
List of Tables

Table 1.1: Clinicopathological features of normal and cancer breast tissues used for β-AR IHC

Table 1.2: Association between β-blocker usage and clinicopathological characteristics in breast cancer patients

Table 1.3: β-blocker usage identified in the retrospective study

Table 1.4: EC50 values for propranolol in normal and malignant breast cell lines

Table 1.5: EC50 values for selective and non-selective β-blockers in SK-BR-3 breast cancer cells

Table 4.1: Different Cell Lines Used to Establish the Ideal Combination (CC50)
List of Figures

Figure 1.1: Overexpression of β-ARs in Breast Cancer........................................17

Figure 1.2: Use of β-blockers is correlated with a reduction in the proliferation rate of Stage I breast cancers.................................................................17

Figure 1.3: Testing the efficacy of propranolol in a prospective window of opportunity breast cancer case study.................................................................18

Figure 1.4: ADRB mRNA expression across a panel of normal and malignant breast cell lines..............................................................................................19

Figure 1.5: β-blockers inhibit the proliferation of breast cancer cell lines...........20

Figure 1.6: Non-selective beta blockers exhibit higher efficacy than selective beta blockers at decreasing breast cancer cell viability........................................21

Figure 2.1: Neoadjuvant treatment of a breast cancer patient with propranolol......32

Figure 2.2: Propranolol decreases the mitogenic potential of breast cancer cells........................................................................................................33

Figure 2.3: Propranolol modulates p53 steady state protein levels and post-translational modifications........................................................................34

Figure 2.4: Propranolol induces apoptosis in breast cancer cells.......................35

Figure 3.1 Effects of Propranolol on cell viability of MDA-MB-231 cells to determine CC_{50} ..................43

Figure 3.2: Propranolol in combination with PND show antagonistic effects on the MDA-MB-231 cell line ........................................................................44

Figure 3.3: Effects of Propranolol on cell viability of MCF-7 cells to determine CC_{50} (DMSO).44

Figure 3.4: Effects of Propranolol on cell viability of MCF-7 cells to determine CC_{50}. (PBS)....45

Figure 3.5: Propranolol in combination with PND show potential synergistic activity on cell viability in the MCF-7 cell line.......................................................45

Figure 3.6: Testing efficiency of PND in combination with Propranolol on cell viability in the MCF-7 cell line...........................................................................46

Figure 4.1 Effects of Propranolol on cell viability of the MCF10-A cell line to determine CC_{50}.................................................................52
Figure 4.2 Propranolol and PND show little to no effect on cell viability in the MCF10-A cell line.

Figure 4.3 Propranolol and PND could potentially cause apoptosis in HCC70 cells.

Figure 4.4 Propranolol and PND reduce cell viability of MDA-MB-468 cells.
Chapter 1: Use of non-selective β-blockers is associated with decreased tumor proliferation in early-stage breast cancer

INTRODUCTION:

Despite great advances in treatment options over the past two decades, breast cancer is still a deadly disease claiming many lives. Approximately 1.7 million women worldwide are diagnosed with breast cancer each year and one third of these women will die of this disease [1]. Breast cancer now represents a quarter of all cancers in women worldwide and is a leading cause of cancer death in less developed countries [2]. This is partly because clinical advances, especially expensive new drugs used to combat the disease, are not reaching women living in these regions [3]. Therefore, identifying more affordable treatment options for economically disadvantaged patients with breast cancer remains highly desirable.

The catecholamines, epinephrine and norepinephrine, stimulate cell membrane-spanning β-ARs and mediate a variety of physiological responses including vascular smooth muscle relaxation, bronchodilation, and other processes responsive to the sympathetic nervous system [4]. Due to their roles in modulating these processes, β-AR antagonists (β-blockers) have been used for decades to manage cardiac arrhythmias, protect against myocardial infarction, and treat hypertension [5]. Recently, several retrospective analyses of data from patients diagnosed with carcinomas have shown note-worthy responses to β-blockade including reduced cancer risk, reduced metastasis, reduced tumor-associated patient mortality, and increased progression free survival [6–15]. In contrast, other studies have found no evidence to suggest that β-blockers prevent cancer occurrence or risk of death [16–18]. Our labs have spearheaded efforts to examine the mechanism of action and clinical use of β-blockers against tumors and have reported that β-blockade disrupts proliferation, survival, migration, cell-to-matrix attachment, and global transcriptional expression patterns in vascular tumors including infantile hemangiomas and
angiosarcomas [19–23]. Other labs have shown that β-AR signaling affects epithelial-to-mesenchymal transition [24] and β-blockade reduces tumor angiogenesis, cell proliferation, migration, and invasion [25–30]. Our labs and others have translated these retrospective and preclinical studies into the clinic to report that use of the β-blocker propranolol in patients with lethal angiosarcomas results in decreased tumor proliferation and sustained therapeutic responses [31–33].

Three β-ARs contribute to β-adrenergic signaling, and each plays distinctive and sometimes overlapping physiological roles largely based on their unique protein expression patterns in the human body [34]. Overexpression of β-ARs has been reported in oral squamous-cell and hepatocellular carcinomas, and their expression has been correlated with cervical lymph node metastasis, age, tumor size, and clinical stage [35, 36]. We hypothesized that the use of β-blockers would result in reduced proliferation rates in breast cancer. Indeed, it has previously been shown using in vitro models that propranolol potentiated the anti-angiogenic and anti-tumor efficacy of chemotherapy agents in breast cancer [29]. We tested this hypothesis by assessing the expression of β-AR1, 2, and 3 in breast carcinoma tissues and performing a retrospective analysis of 404 patients to compare the proliferation rates of breast tumors in patients who had taken β-blockers in the year prior to diagnosis relative to those who had not. We corroborated our retrospective findings using a prospective window of opportunity case study on a breast cancer patient and using in vitro cell based assays on a large panel of established breast cancer cell lines.

Drug repurposing or repositioning is a useful strategy used to find new therapeutic purposes for existing drugs, which can bring clinical treatments out faster at a fraction of the cost of new experimental drugs. Drug repurposing is a trendy topic that has numerous advantages in comparison to new drug development such as, faster access to drugs considering they are already
FDA approved. Numerous existing drugs, which include discontinued and shelved drugs, are being reevaluated for different therapeutic uses to treat common and rare diseases. The development of new anticancer and other therapeutic drugs is often linked with costly price tags and long periods of waiting for conclusive clinical trials.

In the 1960’s Retrovir was studied as a cancer drug and was proven to be unsuccessful [57]. However, in 1987 scientists found that this drug could be useful for treatment of AIDS and therefore, was approved by the FDA for that purpose [57]. Sir James Black organized the efforts to utilize drugs that were already available on the market stating that “The most fruitful basis for the discovery of a new drug is to start with an old drug” [58].

**β AR-1 and β AR-3 are Overexpressed in Breast Cancer**

To determine if β-ARs are aberrantly expressed in breast cancer, immunohistochemistry (IHC) was performed on sections of normal and cancerous breast tissue. In normal breast tissue, β1-AR, β2-AR, and β3-AR staining was observed in both inner luminal epithelial cuboidal to columnar cells and outer myoepithelial contractile cells, yet largely absent in fibro-adipose tissues (Figure 1A). In breast cancer tissue, β-ARs were observed throughout the tumor cells and to a lesser degree within the tumor stroma (Figure 1.1A). IHC intensity scores were collected for each tissue evaluated, revealing that both β1- and β3-AR are expressed at a higher level in breast cancer relative to normal breast tissue. A difference in β2-AR expression was not detected between normal and breast cancer tissue (Figure 1.1B).

**Use of Non-Selective β-blockers is Associated with Reduced Tumor Proliferation in Early-Stage Breast Cancer Patients**

We carried out a retrospective study of 404 patients diagnosed with breast cancer to assess the association between use of β-blockers and breast tumor proliferation rates. No difference was
found in tumor staging or hormone receptor status between users of β-blockers and non-users (Table 1.2, Figure 1.2). However, in patients with Stage I breast cancer, use of β-blockers revealed a significant decrease in the Ki-67 based tumor proliferative index compared to patients who were non-users of β-blockers (p = 0.02) (Table 1, Figure 1.2). In addition, a trend towards a significant (27% decrease; p = 0.1096) association was observed between β-blocker usage and Ki67 index in Stage II breast cancer. A recent publication suggested that only nonselective β-blockers were effective at decreasing mortality in ovarian cancer patients [15], thus we stratified our Stage I breast cancer patients based on β-AR selectivity. A significant decrease in the tumor proliferative index was observed in patients taking non-selective β-blockers (Figure 1.2B) (control=28.9% +/- 2.2 (SEM); non-selective β-blockers = 7.1% +/- 1.1 (SEM); p < 0.0001). This difference was not found in Stage II, III, or IV breast cancer patients.

**Propranolol Reduced the Breast Cancer Proliferative Index in a Window of Opportunity Case Study.**

To prospectively test the findings from our retrospective study, we administered a non-selective β-blocker, propranolol, to a patient treated at the Texas Tech Breast Care Center. The patient had a diagnostic mammogram and ultrasound at baseline, showing a solid micro-lobulated mass with irregular margins at 1 o’clock position, measuring 1 cm in diameter. She presented with an enlarged lymph node in the left axilla (3.5 cm), and ultrasound guided biopsy of the left axillary lymph node revealed no evidence of metastatic carcinoma. Pathology revealed hormone receptor positive, HER2-neu negative invasive ductal carcinoma, 0.5 cm × 1.0 cm × 1.0 cm, moderately differentiated, with negative surgical margins. Based on immunohistochemistry, the tumor was positive for all three β-ARs (Figure 1.3A). Immunohistochemistry for Ki-67 was performed on the diagnostic biopsy (pre-treatment with propranolol) and on the surgical resection (post-treatment; 25 days of propranolol as indicated in the Materials and Methods section). A 23% reduction in the mean Ki-67 proliferative index was observed in the posttreatment tumor (Mean +/- SEM: 10%
+/- 1.0) compared to the pre-treatment tumor (Mean +/- SEM: 13% +/- 0.5) (p = 0.02) (Figure 1.3B). Given that substantial concordance has been reported for the Ki-67 index between breast cancer core needle biopsies and their respective final surgical specimen [37], we believe this propranolol mediated statistically significant decrease in tumor proliferation is valid.

**β- Blockade Reduced the Proliferative Index in Breast Cancer Cell Lines**

While our retrospective analysis revealed that selective β-blockers reduced the proliferative index of breast tumors, it is currently unknown whether these drugs directly affect the malignant cells within the tumor or indirectly regulate tumor proliferation through alternative mechanisms. We performed meta-analysis on a panel of established cell lines to determine the relative mRNA expression of the β-Adrenergic Receptors (ADRBs) in non-tumor versus malignant breast cancer cells. Our data revealed that ADRB1 and ADRB3 mRNA was consistently expressed across all cell lines tested, while ADRB2 mRNA expression exhibited variations from one cell line to another (Figure 1.4). It is worth noting that ADRB2 mRNA is the most highly expressed ADRB gene in ~90% of the cell lines tested. We subjected the panel of breast cancer cells to a dose curve of the non-selective β-blocker propranolol and measured cell viability. A primary culture of HMECs was used as a control. While cell viability of HMECs was minimally affected by even very high concentrations of propranolol (EC50 > 200 µM), cell viability in the panel of breast cancer cells was variably affected by the addition of propranolol with EC50 values ranging from 18 µM to greater than 200 µM depending on the cell line tested (Figure 1.5A, Table 1.4). Ki-67 staining of the SK-BR-3 line revealed that propranolol disrupted breast cancer cell proliferation (Figure 1.5B).

To determine the molecular mechanism by which propranolol decreases breast cancer viability, we performed an antibody array and validation experiments to examine the phosphorylation status of a number of proteins involved in cell proliferation and survival. Our data revealed that 24hour exposure of SK-BR-3 cells to 18 µM propranolol (this cell line’s EC50) resulted in decreased phosphorylation of multiple mitogenic activated protein kinases (MAPKs) as well as the cAMP
responsive element binding protein (CREB), and increased phosphorylation of AKT (PKB, Protein Kinase B), glycogen synthase kinase 3 (GSK3), and p53 (Figure 1.5C and 1.5D). These data suggested that propranolol induced a cellular state indicative of reduced proliferation and increased cell stress/damage. Similar to what was observed in the retrospective patient study described above, neither β1-AR nor β2-AR selective inhibitors, when used alone, were as effective as the non-selective β-blockers propranolol or carvedilol (Figure 1.6A, Table 1.5). It should be noted that nebivolol, a β1-AR inhibitor, was more effective than any of the β-AR selective inhibitors, however this compound also exhibits a large number of off-target effects against serotonin, dopamine, histamine, and α-AR receptors. Additionally, the non-selective β-blocker carvedilol, which also is also a noted α-AR receptor inhibitor, was more effective at reducing breast cancer cell number than propranolol which targets β-AR receptors. Combinations of selective β1-AR and β2-AR inhibitors failed to produce synergy based on two independent models [38, 39] (data not shown), and these combinations, when added at their determined EC50 values for SK-BR-3 cells, also failed to replicate the decreases in viability observed following propranolol treatment.

DISCUSSION:

In this study, we have shown that all three β-ARs are expressed in breast cancer tissue, and β1-AR and β3-AR are overexpressed in breast cancer relative to normal breast tissue. In addition, this study suggests that use of non-selective β-blockers in patients with early-stage breast cancer may lead to decreased tumor proliferation.

Aberrant expression of β2-AR is associated with promoting the oncogenic properties of breast cancer, increasing axillary lymph node metastasis, and leading to poor disease free survival [40, 41]. β2-AR potentially mediates these processes through activating a cAMP-calcium feed-forward loop to drive breast cancer cell invasion and promote invadopodia formation to enhance breast cancer cell invasion [42, 43]. While β2-AR undoubtedly plays an important role in breast
cancer, our data show that β1-AR and β3-AR are expressed more in breast cancer tissue relative to normal mammary epithelium at the protein level. Indeed, β1-AR and β3-AR are reportedly involved in cancer processes, whereby β1-AR has been shown to contribute to enhanced lipolysis in cancer cachexia [44] and β3-AR missense mutations have been correlated with obesity related breast cancer in African-Americans [45]. ADRB mRNA expression was not significantly different between normal and cancer cell lines, suggesting some form of translational or post-translational regulation likely contributes to the differential expression of β1-AR and β3-AR protein levels between the normal and malignant tissues.

A wealth of retrospective reports supports the therapeutic utility of β-blockers in breast cancer and other malignancies [6–15]. While many of these retrospective studies examined therapeutic endpoints such as progression free survival and patient mortality, none have mechanistically correlated tumor response at the molecular level to β-blockade. Preclinical studies in diverse cancers have revealed that β-blockade decreases tumor cell proliferation rates [21, 27, 46–49]. In this study we sought to determine if β-blockade affects tumor cell proliferation in a large patient population stricken with a very common tumor type. Our data showed significant association between the use of non-selective β-blockers and breast cancer proliferation rate, revealing an over 75% reduction in the proliferation rate of Stage I breast cancer following use of non-selective β-blockers such as carvedilol, propranolol, or sotalol. These data suggest a possible use for non-selective β-blockers in breast cancer prevention or in the treatment of early-stage disease before the development of advanced tumors. Moreover, the efficacy of non-selective over selective β-blockers corroborates previously reported data revealing that non-selective β-blockers, but not selective β-blockers, were effective at reducing mortality in ovarian cancer patients [15].
Our in vitro cell line-based data demonstrated that there was no correlation between the sensitivity of the breast cancer cell lines for beta blockade and their respective levels of β-AR mRNA expression. In cell lines we corroborated our retrospective and prospective analyses, showing that non-selective beta blockade was more effective at reducing breast cancer cell viability than selective beta blockade. Based on our expression data, both of the non-selective beta blockers, propranolol and carvedilol, were more effective than any selective beta blocker, and we suspect the increased efficacy of carvedilol over propranolol was based on carvedilol’s added alpha-1 adrenergic (α1-AR) receptor inhibitor activity (Kᵢ = 2.2 nM). Only nebivolol, a third generation β1-AR selective inhibitor, came close to recapitulating the activity of the non-selective beta blockers, however this compound exhibits the highest β1-AR affinity among β-blockers, uniquely stimulates the activity of nitric oxide synthase, and has a number of off-target effects including α1-AR (Kᵢ = 1.2 μM), serotonin 5-HT1a (Kᵢ = 20 nM), serotonin 5-HT2 (Kᵢ = 700 nM), dopamine (Kᵢ = 4 μM), and histamine H1 receptor (Kᵢ = 2.4 μM) which may contribute to its enhanced efficacy relative to other selective β1-AR inhibitors. Indeed, many of these off-target pathways are reportedly novel anti-cancer targets [50–53]. No synergism was calculated based on combinations of β1-AR and β2-AR antagonists, and these combinations were incapable of recapitulating the anti-proliferative effects of the non-selective β-blocker propranolol, suggesting the potential that off target effects partially contribute to the effectiveness of non-selective beta blockers against breast cancer cell lines. As mentioned above, carvedilol exhibits off target effects on β1-AR, and propranolol demonstrates high affinity for serotonin 5-HT1B receptors (Kᵢ = 17 nM) and mild affinity for serotonin 5HT-1D receptors (Kᵢ = 10.2 μM). In summary, future prospective clinical trials testing β-blockers in breast cancer should focus on using non-selective
β-blockers such as propranolol or non-selective α-AR/β-AR inhibitors such as carvedilol against early stage disease.

Our retrospective data analysis has some limitations. First, due to retrospective data collection, we could not determine the long-term effect of using β-blockers. Second, our data comprised only a small proportion of β-blocker users (~13% of the patients). Therefore, we could not adjust for any differences in the baseline cofactors while determining association of β-blockers with Ki-67. Third, we could not collect any other medication data or commodities data which might modify the effect of β-blockers on Ki-67. Nonetheless, our retrospective data analysis along with laboratory experimental studies, prospective study, and bioinformatics meta-analysis study provide ample evidence that non-selective β-blockers could be effective in reducing Ki-67 among early-stage breast cancer. Moreover, interpretation of a single prospective case report must be taken with caution and further clinical studies are needed to corroborate these findings.

If the wealth of preclinical, retrospective, and clinical data continue to support the use of β-blockers as a supplement to existing anti-cancer therapeutic regimes, this class of drugs could be used as safe, low cost therapeutics that are especially useful in the era of prohibitively costly new anti-cancer therapeutics, most particularly in low resource settings where many cancer patients do not possess the financial means or access to expensive novel treatments.

**MATERIALS AND METHODS:**

**Immunohistochemical analysis of β-AR expression in breast tissue**

Immunohistochemistry (IHC) for β1-AR, β2-AR, and β3-AR expression was performed on tissue arrays composed of five normal, fibrofatty breast tissues and twenty invasive, ductal
carcinoma tissues (US Biomax, Cat# MC5003b). The known clinicopathological features associated with these tissues are described in Table 1.4. Sections were deparaffinized, rehydrated, and treated for antigen retrieval using Trilogy (Cell Marque). Nonspecific binding was blocked with background block solution (Cell Marque). The following primary antibodies were used for antigen detection: anti-β1-AR (1:200, Abbiotech Cat# 250919), anti-β2-AR (1:200, Abbiotech Cat# 251604), anti-β3-AR (1:200, Abbiotech Cat# 251604), and anti-Ki-67 (1:200, Abcam Cat# ab15580). Each anti-β-AR antibody was raised against synthetic peptides unique and non-overlapping for each protein. Specificity was tested based on western blotting bands from cancer cell line lysates matching to the appropriate molecular weight for each protein; no overlap in antigenicity was observed (data not shown). Sections were incubated with the CytoScan Alkaline Phos Detection System (Cell Marque) and detected using the DAB substrate kit (Cell Marque). All slides were counterstained with hematoxylin. Immunopositive staining was semi-quantitatively evaluated by 2 individuals blinded to sample identification based on staining intensity within the tumor-positive areas of the tissue.

Retrospective study

Analysis of 404 female patients diagnosed with invasive ductal carcinoma at the Texas Tech Breast Care Center between the years of 2005 to 2014 was performed. 90.1% of the patients were Hispanic. IHC for estrogen receptor (ER), progesterone receptor (PR), Her-2/neu, and Ki-67 tumor proliferative index was available for all patients. Breast cancer clinico-pathological features (age at diagnosis, tumor size, tumor grade, lymph node status, hormonal receptor status) were extracted from each case report, and these data are presented in Table 1. Patients were considered positive for β blocker usage if they had been prescribed β-blockers at any point in the year immediately prior to diagnosis. In total, we identified 54 patients taking β-blockers (61.0 +/- 10 years old) and 350 patients (54.6 +/- 12 years old) that were not taking β-blockers during the
defined time frame. Non-selective β-blockers were prescribed to 22% of the patients, while 78% of the patients were prescribed selective β-blockers (Table 1.5).

**Prospective window of opportunity case study**

A patient diagnosed with Stage I, hormonal receptor positive, HER2-neu negative ductal carcinoma was seen and enrolled in the study at the Texas Tech Breast Care Center. The patient was prescribed 1.5 mg/kg/day propranolol for 18 days and then a tapering dose over the subsequent 7 days. The tapering dose was required by IRB and after consultation with a cardiologist. In total, the patient was administered propranolol for 25 days. The dose of propranolol used in this study was within the typical range of 80–320 mg per day prescribed for ailments such as hypertension, angina pectoris, myocardial infarction, and hemangioma. IHC was performed to confirm the expression of all three β-ARs. Ki-67 immunohistochemistry (1:200, Abcam Cat# ab15580) was performed on tissue sections from the diagnostic biopsy (pre-treatment with propranolol) and on the surgical resection of the tumor (post-treatment with propranolol). The Ki-67 index was blindly quantified in both the preand post-treatment tissues by counting 9 fields under a microscope, with each field composed of 250+ nuclei. The Ki-67 index was calculated as the \( \frac{\text{number of Ki-67 positive nuclei}}{\text{total number of nuclei}} \times 100 \).

**Meta-analysis of breast cancer cell line genomics data**

Quantile normalized mRNA expression (Affymetrix U133+2 arrays) for human breast cancer and immortalized mammary epithelial (HMEL) cell lines were downloaded from the Broad Institute Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle) [54]. The data were processed using GENE-E (The Broad Institute of MIT and Harvard, http://www.broadinstitute.org/cancer/software/GENE-E).
**Viability assays**

Normal breast and breast cancer cell lines were cultured in incubators maintained at 37 degrees Celsius in the presence of 5% CO2. Primary cultures of normal human mammary epithelial cells (HMECs; ATCC #PCS-600–010) were grown in the EBM-2 bullet kit (Lonza) with penicillin/ streptomycin antibiotics. MDA-MB-361 (ATCC #HTB-27) was grown in Leibovitz’s L-15 medium supplemented with 20% fetal bovine serum and penicillin/streptomycin antibiotics. AU565 (ATCC #CRL-2351), HCC70 (ATCC #CRL-2115), BT-549 (ATCC #HTB-122), HCC38 (ATCC # CRL-2314), and MDA-MB-231 (ATCC #HTB-26) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotics. SKBR-3 (ATCC #HTB-30) was grown in McCoy’s medium supplemented with 10% fetal bovine serum and penicillin/ streptomycin antibiotics. All cell lines were used for less than 6 months from the date of purchase and were characterized by ATCC based on morphology, karyotyping, and PCR based approaches to confirm their identify and rule out intra- and inter-species contamination. DL-propranolol hydrochloride (Acros Organics), carvedilol (Tocris Bioscience), esmolol (Santa Cruz Biotechnology), nebivolol (Santa Cruz Biotechnology), atenolol (Acros Organics), butaxamine hydrochloride (Santa Cruz Biotechnology), and ICI 118,551 hydrochloride (Santa Cruz Biotechnology) was added to the cell lines at the indicated concentrations, and cell viability was assessed after 48 hours by alamar blue cell viability assays (ThermoScientific). Two models, the Bliss Independence Model [38] and the Linear Interaction Effect Model [39], were used to evaluate drug combination synergy.

**Immunofluorescence**

SK-BR-3 cells were treated with propranolol for 24 hours and subsequently fixed in 4% paraformaldehyde. Cells were permeabilized with Triton X-100 and incubated with an anti-Ki-67 antibody (1:400, Abcam Cat# ab15580) and anti-rabbit secondary antibodies conjugated to
AlexaFlour 594 dye (ThermoFisher). Hoechst 33342 was used as a nuclear stain. Confocal images were obtained using a Nikon Eclipse Ti.

**Antibody array**

The Phospho-Mitogen-activated Protein Kinase (MAPK) Antibody Array (R&D Systems #ARY002B) was performed on SK-BR-3 cells treated for 24 hours with either a control or 18 µM propranolol according to the manufacturer’s instructions. Data was centered, normalized, and clustered using a centroid linkage via an un-centered similarity metric with Cluster 3.0 software. Heatmaps were visualized using Java Treeview software.

**Western blotting**

Protein extracts from SK-BR-3 cells were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies against phospho-AKT (S473) (Cell Signaling #9271), phospho-p53 (S46) (Cell Signaling #2521), phospho-GSK3β (S9) (Cell Signaling #5558), phospho-p44/42 (ERK1: T202/Y204; ERK2: T185/Y187) (R&D Systems #AF1018), phospho-CREB (S133) (Cell Signaling #9196), and actin (Santa Cruz Biotechnology #sc47778). Proteins were detected with HRP-conjugated secondary antibodies and visualized with the Pierce ECL Western blotting substrate (ThermoScientific) according to the manufacturer’s protocol.

**Statistical analysis**

For statistical analysis of β-AR expression in breast tissue, the Mann-Whitney rank sum test was used. For the retrospective study of breast cancer patient data, the relationship between β-blocker usage and the Ki-67-based proliferative index of the breast tumors was determined with the Mann-Whitney rank sum test. Ki-67 was used to measure tumor proliferation given its established role as a proliferative marker [55], and we have previously demonstrated that the expression of this protein provides a very good concordance with the Recurrence Score Pathology-
Clinical (RSPC value) in breast cancer in our pathology lab [56]. Unpaired t-test was used to compare the age distribution between β-blocker users and nonusers. Comparisons of β-blocker usage to tumor hormonal receptor status and tumor staging were calculated with the Fisher’s exact test. Paired t-tests were used to compare Ki-67 expression in the prospective pre- and post-treatment breast cancer patient sample. Statistical analyses were carried out using STATA 13. Differences were considered statistically significant if the p value was less than 0.05.
Tables:

**Table 1.1: Clinicopathological features of normal and cancer breast tissues used for β-AR IHC**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall</th>
<th>Normal</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td># patient samples</td>
<td>25</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Age [mean years (s.d.)]</td>
<td>48 ± 9</td>
<td>42 ± 7</td>
<td>50 ± 9</td>
</tr>
<tr>
<td>Sex</td>
<td>25F</td>
<td>5F</td>
<td>25F</td>
</tr>
<tr>
<td>Tumor Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>18</td>
<td>N/A</td>
<td>18</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>N/A</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 1.2: Association between β-blocker usage and clinicopathological characteristics in breast cancer patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No β-Blocker N = 349 (86.4%)</th>
<th>β-Blocker N = 55 (13.6%)</th>
<th>p value</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I (# patients)</td>
<td>123 (30.5%)</td>
<td>15 (3.7%)</td>
<td>0.60</td>
<td>No</td>
</tr>
<tr>
<td>Stage II (# patients)</td>
<td>125 (30.9%)</td>
<td>19 (4.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III (# patients)</td>
<td>76 (18.8%)</td>
<td>15 (3.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IV (# patients)</td>
<td>25 (6.2%)</td>
<td>6 (1.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormonal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– (# patients)</td>
<td>95 (23.5%)</td>
<td>17 (4.2%)</td>
<td>0.94</td>
<td>No</td>
</tr>
<tr>
<td>+ (# patients)</td>
<td>249 (61.6%)</td>
<td>38 (9.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown (# patients)</td>
<td>5 (1.2%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– (# patients)</td>
<td>135 (33.4%)</td>
<td>21 (5.2%)</td>
<td>0.89</td>
<td>No</td>
</tr>
<tr>
<td>+ (# patients)</td>
<td>209 (51.7%)</td>
<td>34 (8.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown (# patients)</td>
<td>5 (1.2%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td></td>
<td></td>
<td>1.00</td>
<td>No</td>
</tr>
<tr>
<td>– (# patients)</td>
<td>255 (63.1%)</td>
<td>41 (10.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ (# patients)</td>
<td>58 (14.1%)</td>
<td>9 (2.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown (# patients)</td>
<td>17 (4.2%)</td>
<td>5 (1.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67 index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I Mean (SEM)</td>
<td>28.9 (2.2)</td>
<td>17.2 (4.8)</td>
<td>0.02</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-Selective β-blocker</td>
<td>20.9 (2.2)</td>
<td>20.8 (2.2)</td>
<td>0.25</td>
<td>No</td>
</tr>
<tr>
<td>Selective β-blocker</td>
<td>7.1 (1.1)</td>
<td>&lt; 0.0001</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Stage II Mean (SEM)</td>
<td>39.6 (2.6)</td>
<td>31.4 (6.5)</td>
<td>0.43</td>
<td>No</td>
</tr>
<tr>
<td>Stage III Mean (SEM)</td>
<td>44.9 (3.1)</td>
<td>44.4 (6.1)</td>
<td>0.95</td>
<td>No</td>
</tr>
<tr>
<td>Stage IV Mean (SEM)</td>
<td>38.3 (5.3)</td>
<td>44.0 (6.5)</td>
<td>0.67</td>
<td>No</td>
</tr>
</tbody>
</table>

ER = estrogen receptor.
PR = progesterone receptor.
HER2 = HER2/new receptor.

**Table 1.3: β-blocker usage identified in the retrospective study**

<table>
<thead>
<tr>
<th>β-Blocker</th>
<th># Patients</th>
<th>Dose Range</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvedilol</td>
<td>9</td>
<td>2.12–25 mg daily</td>
<td>Non-selective</td>
</tr>
<tr>
<td>Propranolol Sotalol</td>
<td>1</td>
<td>40 mg daily</td>
<td>Non-selective</td>
</tr>
<tr>
<td>Timolol</td>
<td>1</td>
<td>160 mg daily</td>
<td>Non-selective</td>
</tr>
<tr>
<td>Acebutolol</td>
<td>1</td>
<td>0.25%, 2 drops/day</td>
<td>Non-selective</td>
</tr>
<tr>
<td>Atenolol</td>
<td>1</td>
<td>50 mg daily</td>
<td>β1-AR</td>
</tr>
<tr>
<td>Bisoprolol</td>
<td>12</td>
<td>25–100 mg daily</td>
<td>β1-AR</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>28</td>
<td>25–100 mg daily</td>
<td>β1-AR</td>
</tr>
</tbody>
</table>
### Table 1.4: EC50 values for propranolol in normal and malignant breast cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMEC</td>
<td>&gt; 200 μM</td>
</tr>
<tr>
<td>AU565</td>
<td>81 μM</td>
</tr>
<tr>
<td>BT549</td>
<td>50 μM</td>
</tr>
<tr>
<td>HCC38</td>
<td>93 μM</td>
</tr>
<tr>
<td>HCC70</td>
<td>20 μM</td>
</tr>
<tr>
<td>MDA-MB-175</td>
<td>&gt; 200 μM</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>78 μM</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>&gt; 200 μM</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>18 μM</td>
</tr>
</tbody>
</table>

### Table 1.5: EC50 values for selective and non-selective β-blockers in SK-BR-3 breast cancer cells

<table>
<thead>
<tr>
<th>β-blocker</th>
<th>Selectivity</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>Non-selective</td>
<td>18 μM</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>Non-Selective</td>
<td>11 μM</td>
</tr>
<tr>
<td>Esmolol</td>
<td>β1-AR</td>
<td>&gt; 200 μM</td>
</tr>
<tr>
<td>Nebivolol</td>
<td>β1-AR</td>
<td>32 μM</td>
</tr>
<tr>
<td>Atenolol</td>
<td>β1-AR</td>
<td>&gt; 200 μM</td>
</tr>
<tr>
<td>Butuxamine</td>
<td>β2-AR</td>
<td>144 μM</td>
</tr>
<tr>
<td>ICI-118,551</td>
<td>β2-AR</td>
<td>81 μM</td>
</tr>
</tbody>
</table>
Figures:

Figure 1.1: Overexpression of β-ARs in breast cancer. (A) Representative images of IHC for the β-AR receptors in normal (N = 5) and malignant breast tissue (N = 20). (B) Box and whiskers plot illustrating the expression of β-ARs in the panel of normal and malignant breast tissue.

Figure 1.2: Use of β-blockers is correlated with a reduction in the proliferation rate of Stage I breast cancers. (A) Retrospective analysis of 404 breast cancer patients was performed to determine if use of β-blockers correlated with the tumor Ki-67 index. Box and whiskers plot illustrating the lack of correlation between β-blocker use and breast tumor proliferative rate as determined by the Ki-67 index. (B) Stage I breast cancer patients from the
Figure 1.3: Testing the efficacy of propranolol in a prospective window of opportunity breast cancer case study. (A) Representative 600x images of IHC for the β-AR receptors in a patient diagnosed with Stage I, hormonal receptor positive, HER2-neu negative ductal carcinoma breast cancer. (B) The patient was given propranolol as indicated in the materials and methods section over a 25-day period. Ki-67 IHC was performed on the diagnostic biopsy before propranolol treatment began (pre-treatment) and at the surgical resection after 25 days propranolol (post-treatment). The scatter plot depicts the Ki-67 index pre- and post-treatment across 9 vision fields with over 250 cells/field.
Figure 1.4: ADRB mRNA expression across a panel of normal and malignant breast cell lines. mRNA expression of ADRB1, ADRB2, and ADRB3 across a panel of established breast cancer lines based on data housed in the CCLE database. Expression of the mRNAs in a human immortalized mammary epithelial cell line (HMEL) was used as a control.
Figure 1.5: β-blockers inhibit the proliferation of breast cancer cell lines. (A) A dose curve of propranolol was administered to a panel of breast cancer cell lines and normal primary mammary epithelial cells (HMECS) and viability was assessed. (B) Immunofluorescent detection of Ki-67 protein expression in control or propranolol-treated (18 μM) SK-BR-3 breast cancer cells after 24 hours. Hoechst 33342 was used as a nuclear counterstain. (C) Heatmap depicting phospho-MAPK antibody array results testing the status of 24 kinases in SK-BR-3 cells subjected to 24 hours control or propranolol (18 μM). (red = upregulated; green = downregulated; black = no detectable expression). (D) Western blot confirmation of the antibody array results in SK-BR-3 cells subjected to 24 hours control or propranolol (18 μM), confirming the phosphorylation events identified in the antibody array.
Figure 1.6: Non-selective beta blockers exhibit higher efficacy than selective beta blockers at decreasing breast cancer cell viability. (A) Dose curves of β1-AR or β2-AR selective antagonists were administered to the SK-BR-3 breast cancer cell line and cell viability was assayed and compared to propranolol. (B) Dose curves of β1-AR and β2-AR selective antagonist combinations at their determined EC50 values were administered to the SK-BR-3 breast cancer cell line and cell viability was assayed and compared to propranolol.
Chapter 2: The Beta-Adrenergic Receptor Antagonist Propranolol Alters Mitogenic and Apoptotic Signaling in Late-Stage Breast Cancer

INTRODUCTION:

The beta-adrenergic receptors (β-ARs) are a class of G-protein coupled receptors (GPCRs) that are targets of the catecholamines epinephrine and norepinephrine. Stimulation of these receptors induces sympathetic nervous system responses, most notably the fight-or-flight reaction. β-ARs were first detected in breast cancer nearly thirty years ago [59, 60], and it has recently been reported that β-ARs are over-expressed in breast cancer relative to non-diseased breast epithelium [61]. Pre-clinical studies using breast cancer cell lines have correlated β-AR antagonist (beta blocker) treatment to decreased cell proliferation and migration [61-67]. Studies using animal tumor models have yielded similar results, with β-AR antagonists resulting in decreased breast cancer growth, reductions in metastasis to the brain, bone, and other organs, and improved host survival [64,68,71]. Moreover, combinations of β-AR antagonists with standard anti-cancer treatments exhibit improved pre-clinical results for breast cancer compared to single treatments [63,72]. Several retrospective studies have sought to correlate the use of β-AR antagonists with clinical outcomes in breast cancer. In these studies, β-AR antagonists were associated with reduced tumor proliferation rates, decreased mortality rates, decreased metastatic development, longer disease free survival, and reduced tumor recurrence [61,70,72,74]. In contrast, one retrospective study has reported that use of β -AR antagonists has no effect on breast cancer patient survival [75]. In prospective clinical settings, the non-selective β-AR antagonist propranolol significantly reduced the tumor proliferation rate of early stage breast cancer [61] and improved immune biomarker profiles when combined with COX-2 inhibitors [76]. The mechanisms by which beta blockers inhibit the proliferation rate of breast cancer cells and potentially improve clinical outcomes are largely unknown, however a handful of studies have provided some insight into these processes. Propranolol treatment of breast cancer cell lines decreases MAPK, HSP70, and inducible nitric oxide synthase activity, as well as increases interleukin 10 and RANKL expression.
Propranolol exerts effects on breast cancer metabolism through post-transcriptional downregulation of hexokinase 2 to inhibit glucose metabolism [77]. Hexokinase 2, an enzyme often overexpressed in tumor cells, plays a critical role in tumor initiation and development, and leads to negative clinical outcomes [78,79]. Norepinephrine-mediated β-AR stimulation in breast cancer cells enhances cancer cell adhesion to microvascular endothelial cells [80], suggesting that this signaling pathway may play a role in the early steps of the metastatic cascade through interactions with the vascular system. Supporting this possibility, β-AR antagonism inhibits capillary network formation and greatly exacerbates the anti-angiogenic effects of paclitaxel [63].

In the current study, propranolol was prospectively administered to a late-stage breast cancer patient in a neoadjuvant setting. Changes in tumor cell proliferation rates, mitogenic markers, and apoptotic markers were evaluated before and after β-AR antagonist administration. Propranolol mediated alterations in tumor biomarkers were corroborated at the molecular level using a breast cancer cell line.

**Neoadjuvant Treatment of a Late-Stage Breast Cancer Patient with Propranolol**

We have previously reported that administration of propranolol to an early-stage breast cancer patient resulted in a decreased tumor proliferative index [61]. To prospectively evaluate if beta blockade could reduce the proliferation rate of a late-stage breast tumor in a neoadjuvant setting, propranolol was administered to a patient diagnosed with a 6.5 cm invasive lobular carcinoma that was estrogen and progesterone receptor positive, HER2-neu negative, and expressed β1-AR, β2-AR, and β3-AR. The treatment regime is outlined in figure 2.1.

**Propranolol Alters Breast Cancer Cell Cycle Progression**

IHC for Ki-67 was performed on the diagnostic biopsy (before treatment with propranolol) and on the tumor tissue from the radical mastectomy (after 25 days of propranolol treatment). The Ki-67 proliferative indices at the time of diagnostic biopsy and surgical removal were $68 \pm 3\%$.
(mean ± SEM) and 25 ± 2% (mean ± SEM), respectively (Fig. 2.2 A and B). This equated to an approximately 2.7-fold reduction in tumor proliferative index after propranolol treatment (p < 0.0001). Substantial concordance has been reported for Ki-67 indices between diagnostic biopsy and surgical resection [83], therefore an alteration in tumor proliferation, as observed in this patient, can be attributed to propranolol administration with reasonable confidence. We corroborated this finding using the MDA-MB-231 breast cancer cell line, which was originally isolated from a metastatic pleural effusion. Cell cycle analysis was performed using flow cytometry in control and propranolol (40 mM) treated breast cancer cells (2 h treatment, as substantial changes in cell viability were visually observed at this time). In our previously published study examining the effects of beta blockade on MDA-MB-231 cells, we demonstrated that the EC50 for propranolol in this cell line was approximately 78 mM [61]. In the current study, we treated MDA-MB-231 cells with a lower dose of propranolol for multiple reasons: 1) the EC50 of propranolol established in our previous publication is not a physiologically attainable plasma concentration in patients; 2) dosing of patients with chemotherapeutic compounds generally involves low dose/longer treatment regimens that are not well recapitulated in lethality testing associated with in vitro cell culture systems; and 3) we sought to examine the acute molecular effect of sub-lethal doses of propranolol on the breast cancer cells. The anthracycline chemotherapy drug, doxorubicin (3 mM), was used as a positive experimental control for this and all subsequent in vitro experiments. Addition of propranolol reduced the percentage of the cell population residing in the G2/M phase of the cell cycle (Fig. 2.2C and D), and increased the sub-G1 cell population, representing dead or dying cells (Fig. 2.2C and E). Moreover, the expression of multiple cyclins was reduced following 24 h propranolol treatment (Fig. 2.2F and G), further demonstrating the impact of β-AR antagonism on breast cancer cell proliferation.
Propranolol Enhances p53 Activation in Breast Cancer Cells

IHC for total p53 was performed on the diagnostic biopsy and tumor tissue from the radical mastectomy in the late-stage breast cancer patient. The p53 indices at the time of diagnostic biopsy and surgical removal were 8 ± 2% (mean ± SEM), and 20 ± 2% (mean ± SEM), respectively (Fig. 2.3A and B). This equated to an approximately 2.5-fold increase in p53 protein expression after propranolol treatment (p < 0.0001). These data were corroborated by immunofluorescent staining of MDA-MB-231 cells for total p53, revealing a marked increase in p53 protein levels by 6 h post-treatment with propranolol (Fig. 2.3C). Immunoblotting of the breast cancer cells showed similar propranolol-mediated increases in total p53 levels between two to 6 h post-treatment (Fig. 2.3D). Using immunoblotting, we evaluated specific post-translational modifications that occur on the p53 protein following 6 h propranolol treatment of MDA-MB-231 cell lysates. Phosphorylation events were normalized based on levels of actin and total p53, revealing that propranolol increased the phosphorylation of p53 on residues S9, S37, and S46 (Fig. 2.3E and F).

Propranolol Induces Apoptosis in Breast Cancer Cells

IHC for the pro-survival protein, Bcl-2, was performed on the diagnostic biopsy and tumor tissue from the radical mastectomy in the late-stage breast cancer patient. Decreased Bcl-2 staining intensity was observed after propranolol administration. We then treated MDA-MB-231 cells with a time course of propranolol up to 6 h and evaluated changes in cleaved caspase levels using immunoblotting. Our analysis revealed that propranolol markedly increased the levels of caspase 3, 6, and 9 cleavage products as early as 2 h post treatment (Fig. 2.4B and C). MDA-MB-231 cells treated for 6 h with control or propranolol were stained with the apoptotic and necrotic markers, Apopxin Green Indicator and 7-AAD, respectively, revealing substantial apoptotic increases in propranolol treated cells (Fig. 2.4D). At the time point evaluated, we observed no visible increases in necrosis for the propranolol treated cells (Fig. 2.4D).
DISCUSSION:

In a retrospective analysis of over 400 patients, we previously reported that use of non-selective β-AR antagonists was associated with decreased proliferation of early-stage breast cancer patients [61]. While we did not observe an effect on late-stage breast cancer patients in this study, these observations may have been confounded due to an inability to control β-AR antagonist dosing or length of administration in a retrospective setting. Therefore, we sought to evaluate, in a controlled prospective manner, whether propranolol could inhibit tumor growth in a late-stage breast cancer patient. Our findings revealed that neoadjuvant propranolol reduced the proliferative index of a stage III breast tumor by 2.7-fold. We then corroborated these findings at the molecular level, showing that propranolol disrupted the cell cycle distribution of breast cancer populations and altered the protein expression of the cell cycle regulatory proteins cyclin A, D1, E1, and E2. Decreased proliferation in response to β-AR antagonists has been reported across multiple tumor cell types, and is accompanied by disruption of mitogenic signal transduction cascades including AKT, MAPK, and cAMP [61,84-87]. These β-AR antagonist-mediated changes in tumor proliferation rates and mitogenic signaling cascades may contribute to decreased breast cancer metastasis and recurrence, as well as improved overall patient survival demonstrated in a number of clinical studies [70,72,74,88-90]. In addition to decreased Ki-67 antigenicity, we observed an increase in pro-apoptotic p53 protein expression and a decrease in pro-survival Bcl-2 protein expression in late stage breast cancer tissue following neoadjuvant propranolol administration. Moreover, β-AR antagonism increased the sub-G1 cell cycle population of breast cancer cells, suggesting that blocking β-AR signaling leads to breast cancer cell death. Indeed, our live/dead assay indicated that propranolol enhanced apoptosis as indicated by higher Apopxin staining in propranolol treated cells relative to the control. We corroborated these findings at the molecular level by showing that propranolol stabilized p53 protein levels in breast cancer cells and enhanced the phosphorylation of p53 regulatory sites including S9, S37, and S46. Stabilization of p53 by DNA damage or cell stress has been extensively shown to trigger multiple anti-proliferative and
pro-apoptotic signaling cascades [91]. DNA damage induces phosphorylation of p53 at S9 and S37, while phosphorylation at the S46 residue regulates the ability of p53 to induce apoptosis [92,93]. Moreover, propranolol led to increased levels of cleaved initiator caspase 9 and execution caspases 3 and 6 in breast cancer cells, further indicating that propranolol treatment leads to programmed cell death. Similar effects on cell survival and apoptotic signaling have been observed in previous studies across other tumor types, including propranolol-induced caspase activation, increases in p53 steady state protein levels, and enhanced cell death [86,94,98]. We have previously reported propranolol selectivity against breast cancer cells, while this treatment relatively spares non-diseased mammary epithelial cells [61]. Our understanding of the mechanism behind selectivity of propranolol is currently very limited, whereas mechanism(s) have been addressed to some degree in infantile hemangiomas. While it has been reported that propranolol inhibits cell proliferation of both infantile hemangioma and normal endothelial cells in culture [85], selectivity has been observed in infantile hemangioma cells. β -AR antagonism induces apoptosis in hemangioma endothelial cells yet spares hemangioma-derived stem cells in part through a differential regulation of Akt and other survival regulators [99], as well as a propensity for propranolol to selectively promote adipogenesis in hemangioma-derived stem cells [100]. Further studies are necessary to understand the mechanisms behind the selectivity of β -AR antagonism against diseased cells. While the current study focused exclusively on the effects of propranolol on mitogenic and apoptotic signaling regulators in breast cancer, studies from other tumor types suggest that β -AR antagonism may disrupt a variety of processes. For instance, propranolol interferes with several steps during angiogenesis including cell proliferation and formation of capillary tubules [101,102]. β -AR antagonism also modulates the expression and activation of angiogenic signaling pathways including angiopoietin/TIE2, vascular endothelial growth factor (VEGF), and hypoxia inducible factor (HIF) [85,86,96,103-105]. Propranolol exhibits a biphasic effect on vascular resistance, with lower (10-20 mg/kg/day) and higher (20+ mg/kg/day) doses inducing vasoconstriction and vasodilation, respectively [106]. This same study showed that propranolol affects tumor arteriogenesis, but not capillary density in melanoma tumor
models [106]. In both breast and neuroblastoma models, propranolol has been shown to increase the treatment efficacy of chemotherapy by potentiating anti-angiogenic effects [63], suggesting that combination treatments with propranolol may be more effective at decreasing vascular networks than either treatment alone. An emerging area of research where β-AR 162 biomedical journal 42 (2019) antagonism may substantially impact the oncogenic process is regulation of tumor immunity. Catecholamine activation of β-ARs has long been known to suppress innate and cellular immunity by decreasing interleukin (IL)-12 production and increasing IL-10 secretion, thus shifting the immune reaction away from a beneficial T helper cell 1 (TH1) response [107,108]. A recent study in melanoma has demonstrated that propranolol is capable of reducing infiltration of immunosuppressive myeloid cells into the tumor and increasing infiltration of cytotoxic lymphocytes [109]. As the ability of tumors to foster an immunosuppressive microenvironment is a major impediment to current immunotherapy strategies, the addition of propranolol with this class of anti-cancer therapeutics may help shift the balance toward a more desirable immunoreactive state within the tumor.

**MATERIALS AND METHODS:**

**Neoadjuvant treatment of a late stage breast cancer patient with propranolol**

Clinical studies presented in this report were performed following approval of the Texas Tech University Health Sciences Center Institutional Review Board. A female patient, age 44, presented at the Texas Tech Breast Care Center with a stage IIIA, 6.5 cm left breast invasive lobular carcinoma (T3N1M). Tumor tissue from the diagnostic biopsy was collected for analysis. The patient was immediately prescribed 1.5 mg/kg/day propranolol for 18 days and then a tapering dose over the subsequent 7 days. After the treatment period, the tumor was removed through a radical mastectomy and tumor tissue was collected for analysis.
Immunohistochemistry (IHC)

Tumor tissue was collected and processed from both the patient's diagnostic biopsy (pre-treatment) and the radical mastectomy (post-treatment). IHC for Ki-67 was performed at the University Medical Center's Pathology facilities according to their in-house procedures. The Ki-67 index was blindly quantified in both the pre- and post-treatment tissues by counting 10 fields under a microscope, with each field composed of 250 nuclei. The Ki-67 index was calculated as the (number of Ki-67 positive nuclei/total number of nuclei) 100. For IHC staining of total p53 and Bcl-2 proteins, slides were deparaffinized, rehydrated, and stained using the rabbit specific HRP/DAB (ABC) Detection IHC Kit (ab#64261) or the mouse specific HRP/DAB (ABC) Detection IHC Kit (ab#64259) according to the manufacturer's protocols. Antibodies included: anti-p53 (total) (Cell Signaling #2527) and anti-Bcl-2 (Abcam #ab196495).

Cell culture and treatment

MDA-MB-231 breast cancer cells (ATCC #HTB-26) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were maintained in a water-jacketed CO2 incubator at 37°C. DL-propranolol hydrochloride (Acros Organics) and doxorubicin hydrochloride (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and were administered as indicated for each experiment. As a solvent control, DMSO was tested at the same concentration (1% v/v) as contained in the experimental samples. Also, untreated cells were used to establish the background of dead cells provoked by factors inherent to the cell cultures and cell manipulation.

Flow cytometry

MDA-MB-231 cells were collected via trypsinization and resuspended in PBS. Nuclear isolation medium (NIM)-DAPI solution (Beckman Coulter) was added to the cell suspensions and analyzed by a Gallios flow cytometer (Beckman Coulter) according to previously described
methods [23]. Determination of the main phases of the cell cycle subpopulations (G0/ G1, S, and G2/M), as well as the occurrence of cells experiencing DNA fragmentation (sub-G0/G1; apoptotic subpopulation), was performed as previously described [23]. The percentage of each cell cycle phase distributions was determined by using Kaluza flow cytometry software (Beckman Coulter). Consistently, twenty thousand cells were measured in each condition.

**Immunoblotting**

Lysates were subjected to SDS-PAGE, and transferred to polyvinylidene fluoride membranes using the Trans-Blot Turbo Fig. 1 Neoadjuvant treatment of a breast cancer patient with propranolol. (A)Immunohistochemical staining of breast cancer tissue from the patient discussed in this study for b1-AR, b2-AR, and b3-AR proteins. Purple/brown staining indicated positivity for the antigen tested. (B) Flow diagram showing the treatment and tumor tissue collection schemes for the stage III breast cancer patient. biomedical journal 42 (2019) 155 e165 157 Transfer System (Bio-Rad). Membranes were blocked in Trisbuffered saline plus 3% bovine serum albumin and 0.05% Tween-20, and incubated with the following antibodies as indicated for each experiment: anti-cyclin A (Cell Signaling #4656), anti-cyclin B1 (Cell Signaling #4138), anti-cyclin D1 (Cell Signaling #2978), anti-cyclin D2 (Cell Signaling #3741), anti-cyclin E1 (Cell Signaling #4129), anti-cyclin E2 (Cell Signaling #4132), anti-p53 (total) (Cell Signaling #2527), antiphospho-p53 (S9) (Cell Signaling #9288), anti-phospho-p53 (S15) (Cell Signaling #9286), anti-phospho-p53 (S20) (Cell Signaling #9287), anti-phospho-p53 (S37) (Cell Signaling #9289), anti-phospho-p53 (S46) (Cell Signaling #2521), antiphospho-p53 (S392) (Cell Signaling #9281), cleaved caspase 3 (Cell Signaling #9664), cleaved caspase 6 (Cell Signaling #9761), cleaved caspase 9 (Cell Signaling #7237), and anti-beta actin (Santa Cruz Biotech #sc8432). Each primary antibody was detected with an appropriate 1:1000 HRP-conjugated secondary antibody, subjected to Supersignal West Dura Extended Duration Substrate (ThermoFisher Scientific), and digitally captured using a GE Image Quant Las4000 imaging system.
**Immunofluorescence**

Cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and incubated with the anti-p53 antibody (Cell Signaling #2527), rhodamine-conjugated phalloidin (Cytoskeleton Inc), or Hoechst 33342 (Thermo Fisher Scientific). Appropriate fluorescent-conjugated secondary antibodies were used for primary antibody detection. Single plane high-resolution digital fluorescent images were captured using an LSM 700 confocal microscope (Zeiss) equipped with a 40x immersion oil objective and assisted with Zen 2009 software (Zeiss); for each channel, 1-airy unit (AU) pinhole setting was utilized as previously described [24]

**Live/dead staining**

Cells were stained with Apopxin Green Indicator, 7-AAD, and CytoCalcein Violet 450 using the Apoptosis/Necrosis Detection Kit (Abcam #ab176749) according to the manufacturer's instructions.

**Statistical analysis**

The Mann-Whitney rank sum test was performed in GraphPad Prism version 7 for [Figs. 2B, G, and 3B, F]. One-way ANOVA was performed in GraphPad Prism version 7 for [Figs. 2D, E, and 4C]. Differences were considered statistically significant if the p < 0.01.
Figures:

![Figures](image)

**Figure 2.1: Neoadjuvant treatment of a breast cancer patient with propranolol.** (A) Immunohistochemical staining of breast cancer tissue from the patient discussed in this study for b1-AR, b2-AR, and b3-AR proteins. Purple/brown staining indicated positivity for the antigen tested. (B) Flow diagram showing the treatment and tumor tissue collection schemes for the stage III breast cancer patient.
Fig. 2.2 Propranolol decreases the mitogenic potential of breast cancer cells. (A & B) IHC and graphical representation for Ki-67 performed on tissues from the diagnostic biopsy (pre-treatment) and surgical resection (post-treatment). Brown staining indicates Ki-67 antigenicity. Ten random vision fields were counted per condition. (C & D) DAPI staining of MDA-MB-231 breast cancer cells was analyzed via flow cytometry after treatment with vehicle control or with 40 mM propranolol for 24 h. Three mM doxorubicin was added as a positive experimental control. (E) Quantification of the sub-G1/G0 cell sub-population from the flow cytometry analysis. Cell cycle populations (sub-G1, G1, S, G2/M) are marked in red in the control flow cytometry graph. (F & G) Immunoblotting and quantification of cyclin protein levels in MDA-MB-231 cells treated with vehicle control or 40 mM propranolol. Three mM doxorubicin was added as a positive experimental control. For quantification, cyclins were normalized relative levels of actin. AU indicates arbitrary units.
Fig. 2. 3 Propranolol modulates p53 steady state protein levels and post-translational modifications. (A & B) IHC and graphical representation for p53 protein performed on tissues from the diagnostic biopsy (pre-treatment) and surgical resection (post-treatment). Brown staining indicates p53 antigenicity. Ten random vision fields were counted per condition. (C) Immunofluorescent staining for p53 (green), Hoechst 33342 nuclear counterstain (blue), and rhodamine-conjugated phalloidin as an actin counterstain (red) in MDA-MB-231 cells treated with vehicle control or 40 mM propranolol. Three mM doxorubicin was added as a positive experimental control. (D) Immunoblotting for p53 protein levels in MDA-MB-231 cells treated with 40 mM propranolol over a 6 h time course. Three mM doxorubicin was added as a positive experimental control. (E & F) Immunoblotting of p53 phosphorylation events in MDA-MB-231 cells treated with vehicle control or 40 mM propranolol for 6 h. Three mM doxorubicin was added as a positive experimental control. For quantification, phospho-p53 was normalized relative levels of both total p53 and actin. AU indicates arbitrary units.
Fig. 2.4 Propranolol induces apoptosis in breast cancer cells. (A) IHC for Bcl-2 protein performed on tissues from the diagnostic biopsy (pre-treatment) and surgical resection (post-treatment). Brown staining indicates Bcl-2 antigenicity. (B & C) Immunoblotting of cleavage caspase products in MDA-MB-231 cells treated 40 mM propranolol over a 6 h time course. For quantification, cleaved caspase products were normalized relative levels of beta-actin. AU indicates arbitrary units. (D) MDA-MD-231 cells were treated with vehicle control, 40 mM propranolol, or 3 mM doxorubicin as a positive control for 6 h. Cells were stained with the apoptotic marker Apopxin Green Indicator (green), the necrotic marker 7-AAD (red), and CytoCalcein Violet (blue) as a nuclear counterstain. Images were collected using confocal microscopy.
Chapter 3: Identifying Potential Synergistic Effects of Propranolol and PND on Breast Cancer Cell Lines

INTRODUCTION:

Combination therapies using potential anti-cancer reagents have numerous advantages, such as overcoming drug resistance and eliminating unpleasant cytotoxic side effects. In finding different ideal combinations, different drug reactions can occur. Three main types of chemical reactions that can occur are referred to as pharmacodynamics include antagonism, additive, and synergism reactions. The first reaction, antagonism, is where two or more agents in combination have an overall effect less than the sum of their individual effects. A form of antagonism referred to as pharmacokinetic, is where one drug alters the absorption, distribution, metabolism, or excretion of the other drug. An additive reaction is where two or more chemicals combine and yield a total effect equal to the sum of the effects of each individual chemical, and synergism, where two or more chemicals combine to produce a chemical with a total effect that is greater than the sum of the effects of each individual chemical [129,130]. Synergistic effects can maximize therapeutic effects and minimize side effects.

Numerous therapies used to treat microorganism infections often utilize more than one reagent for treatment, such as the treatment for HIV infection, which is a tri-therapy (three reagents are used). Presently only a few combination therapies are approved for the treatment of certain cancers [131]. Some studies have shown that different types of proposed chemotherapies can utilize synergistic activity of reagents such as, cisplatin and doxorubicin for the treatment of lung cancer; and docetaxel and vinorelbine have shown promising signs in lymphoblastoid cells [131,132]. Our previous data suggests that Propranolol alters cell signaling in early and late-stage breast cancer, therefore we want to test if propranolol, in combination with another therapeutic reagent. Our lab has previously shown that Pyronaridine has anti-cancer properties.
and for this reason we have chosen to combine propranolol and Pyronaridine to test for positive cellular interactions on cell viability.

In the 1970’s Pyronaridine (PND) is a benzonaphthyridine derivative that was synthesized at the Institute of Chinese Parasitic Disease and has been successfully used for treating malaria patients for over 30 years. [133]. PND is believed to be a DNA topoisomerase inhibitor and has shown potent anticancer properties [134]. It has been reported that PND inhibits β- hematin formation and could possibly work as a DNA topoisomerase II [134]. The findings from our lab suggest that PND induces apoptosis and interferes with cell cycle progression in seventeen different cancer cell lines, seven of which were from female breast tissue [134]. Recent experiments from our lab [134] have shown PND is powerful as a single agent, but we want to determine if propranolol and PND could work together rather than working in conjunction with a chemotherapy reagent such as tamoxifen or doxorubicin which are linked to unpleasant side effects. The Villanueva paper [134] showed that PND induces apoptosis via mitochondrial depolarization and because propranolol interferes with mitogenic signaling these two drugs could have very promising effects when used together.

The MDA-MB-231 Cell Line Exhibits Antagonistic Effects

The MDA-MB-231 cell line is derived from a metastatic mammary adenocarcinoma from a 51-year-old patient. This cell line is a triple negative breast cancer (TNBC), meaning it lacks estrogen, progesterone, and human epidermal growth receptor-2 (HER-2), which makes it highly aggressive, with poor prognosis, and very invasive. TNBC accounts for about 10-15% of all breast cancer cases. Because these cells are highly aggressive and invasive, they have become the target of many studies. To determine the cytotoxic concentration (CC50) of this combination the InCell-Analyzer was used. This qualitative method is a laser based high-content imaging
system that allows for differentiation of live and dead cells through a two-dye system; one marking live cells while the other marks dead/dying cells [56]. MDA-MB-231 cells were treated with an array of propranolol doses and the results obtained showed the CC50 for propranolol at 24 hours was around 37uM while PND (Figure 3.1) was around 1.6uM (previously determined by our lab) [134]. To determine the ideal combination, doses of propranolol used ranged from 60uM-10uM, while the dose of the PND remained constant throughout, 1.6uM. Propranolol was added for the first 24 hours followed by the treatment of PND added for the remaining 24 for a total of 48 hours. Both Propranolol and PND play a role in disrupting mitochondrial formation, increasing levels of caspase 3, and ultimately trigger apoptosis, therefore my hypothesis is that together these drugs would work together to inhibit cellular proliferation and viability.

The results indicate that while propranolol and PND both seem to be highly effective independently, when these two reagents are combined, there is no synergistic activity (Figure 3.2). At the highest dosage of propranolol (60uM) in combination with PND (1.6uM) only about 20% cell death is observed. As the dosages of propranolol decrease cell viability begins to increase. The CC50 of propranolol on the MDA-MB-231 cells was determined to be 37uM, but the data reveals that cells treated at 40uM concentration plus PND, yield only 16% of cell death; this suggests that these two drugs may interfere with the activity of one another. This theory could be due to a few reasons such as resistance one of the reagents could interfere in the physiological changes of the cancer cell that ultimately reduces the efficacy of the second drug, or one drug alters the absorption and/or metabolism, of the other drug [135]. One study demonstrated that under prolonged drug exposure, some cancer cells enter a drug tolerant state [131,132]. This sensitivity to subsequent drugs is hard to predict and further studies defining biomarkers should be evaluated. The paper by Loria et al states that when drugs acting on the
same target are administered, the first drug can induce target deregulation or mutation that causes escape from therapy and cross–resistance to the subsequent drug [136]. In another paper published by A J Wood et al, they state that the co- administration of propranolol with other drugs could result in either propranolol induced changes in the disposition of other drugs or possibly an effect of the other drug on the pharmacokinetics of propranolol [130].

**Propranolol and PND Show Potential Synergistic Activity in MCF-7 Cells**

To further assess possible synergistic activity, a different breast cancer cell line, MCF-7 was studied. The MCF-7 cell line is derived from the metastatic adenocarcinoma of a 69-year-old patient. Unlike the MDA-MB-231 breast cancer cell line, MCF-7 is estrogen receptor (ER) and progesterone receptor (PR)-positive. Hormone receptors play a pivotal role in cancer progression as well as possible treatments. Estrogen has also been implicated in playing a key role in cancer survival and metastasis.

Using the InCell-Analyzer to determine the CC50 of propranolol on this cell line, it was revealed that the MCF-7 has a relatively high sensitivity to DMSO (Figure 3.3). The DMSO control has a considerable number of dead cells, compared to what we would normally anticipate. To reassess the CC50 and eliminate bias, the experiment was redone with the propranolol dissolved in PBS instead of DMSO and after 24 hours the CC50 was concluded to be around 35uM. (Figure 3.3). Two sets of data were then generated. The first set is the MCF-7 cells treated with different doses of Propranolol for the first 24 hours followed by the treatment with PND at the CC50 (1.6uM; also included in the data for reference) for the last 24 hours (Figure 3.4). The next set of data is the cells treated with different doses of PND for the first 24 hours followed by the treatment of Propranolol at the CC50 (35uM also included in data for reference) (Figure 3.5).
After 48 hours the results of the MCF-7 cells differ drastically from the MDA-MB-231 treated cells. Interestingly the different treatments also yielded different results. Treatment with propranolol for the first 24 hours followed by PND showed that at higher concentrations (120uM, 100uM, and 80uM), almost total cell death was achieved (Figure 3.4). Cell viability begins to increase to around 15%, at concentrations of 60uM, whereas the MDA-MB-231 cells treated at 60uM, 80% viability was observed. As the concentrations lower from 40uM to 10uM cell viability begins to increase. The most effective combination that induces a response halfway between the baseline and maximal is 10uM Propranolol + 1.6uM PND. (Figure 3.5).

The cells that were treated with different doses of PND for the first 24 hours showed that around 8uM of PND the viability is approximately 5%, as the dosages lower the more viability is seen, but is still significantly low (about 30%) (Figure 3.6). Interestingly the cells treated with Propranolol at 40uM (close to the CC50 37uM) and PND at 1.6uM cell death is around 75%. (Figure 3.5), whereas the cells that received the treatment of PND at 2uM (close to the CC50 1.6uM) and Propranolol at 37uM (CC50) 85% cell death is achieved (Figure 3.6). The data would suggest that the sequencing of potential cancer treatment regimens may be critical in determining the effectiveness in reducing cellular proliferation.

**DISCUSSION:**

The data presented in this study from both the MDA-MB-231 and MCF7 cell lines differ significantly. The MDA231 cell line seemed to be relatively unaffected by the treatment of propranolol and PND, while the MCF7 showed good responsiveness. At the highest dosage of propranolol and PND only about 20% cell death was observed in the MDA-MB-231 cell line. Although the CC50 of propranolol was determined to be 37uM, the data reveals that cells treated
at 40uM concentration yield only about 16% of cell death. On the other hand, MCF7 cells showed when they are treated with 60uM propranolol and 1.6uM PND, cell viability is at about 15%, whereas 80% cell viability was observed in the MDA-MB-231 cells treated at 60uM propranolol and 1.6uM PND. Interestingly the cells treated with Propranolol at 40uM (close to the CC50 37uM) and PND at 1.6uM cell death is observed at around 75%, while the MCF-7 cells that received the treatment of PND at 2uM (close to the CC50 1.6uM) and Propranolol at 37uM (CC50), 85% cell death is detected. The most effective combination in terms of reducing cell viability by 50% for the MCF-7 cells was noted to be < 10uM Propranolol + 1.6uM PND and < .5uM PND + 35uM Propranolol.

It is hard to identify the exact reason why additive effects are seen with the MCF7 cells, while antagonistic effects are seen with the MDA-MB-231 cell line. It is likely that different treatments that work better for different cancers are not effective on others due to different genetic mutations as well as morphological features. A few reasons why treatments can be seen to work in some cell lines might be due to mutations of the drugs molecular target, changes in the way the drugs are interacting with each other, changes in the cell cycle that alter cell signaling pathways, changes due to alteration in absorption, and even ATP binding cassette (ABC transporters) expelling the chemotherapy drugs [137, 138].

Moving forward, we will test additional breast cancer cells to try to better understand if the same variability of effects is seen in other cancer cells. Using different types of breast cancer cell lines could shed light onto the possible different pathways being targeted that can lead to synergistic activity. This data can be useful for future drug screening work to determine if single cancer drug therapies or combinations with other potential therapies can lower not only the
dosages of the reagents, and eliminating unpleasant side effects, but could also potentially be more cost effective.

**MATERIALS AND METHODS:**

**Cell culture and treatment**

MDA-MB-231 breast cancer cells (ATCC #HTB-26) cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. MCF-7 breast cancer cells were also cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were maintained in a water-jacketed CO2 incubator at 37°C. DL-propranolol hydrochloride (Acros Organics) and doxorubicin hydrochloride (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and were administered as indicated for each experiment. As a solvent control, DMSO was tested at the same concentration (1% v/v) as contained in the experimental samples. Also, untreated cells were used to establish the background of dead cells provoked by factors inherent to cell cultures and cell manipulation. After the first round of experiments, it was shown that the MCF-7 cell lines are sensitive to DMSO, therefore another set of DL-propranolol hydrochloride (Acros Organics) was dissolved in phosphate-buffered saline (PBS).

**Live/dead staining Differential nuclear staining assay to quantify cell death**

To analyze the potential cytotoxic activity of Propranolol and PND, the Differential Nuclear Staining (DNS) assay, live-cell bio-imaging was utilized. For this assay, cells were seeded at 10,000 cells/well density in a 96-well plate in 100μl of culture media, incubated overnight, and treated with a gradient of Propranolol for the first 24hrs and PND at 5μM for the last 24 hrs for a total of 48hrs incubation. Hoechst 33342 and Propidium iodide, which are two fluorescent nucleic acid intercalators were added to each well two hours prior to analysis; at a
final concentration of 1 μg/ml each. Due to its high permeability, Hoechst stains all the cells (total dead and alive), whereas PI only stains dead or dying cells. Montages of 2 by 2 images were captured directly from each individual well of the culture plates by using a multi-well plate reader IN Cell 2000 analyzer. (31). The following controls were included in every single plate: PBS and DMSO as solvent/vehicle control, Hydrogen peroxide as a positive control for cytotoxicity, and untreated cells to determine the background of toxicity due to cell manipulation and intrinsic factors usually associated with the culture protocol. Each experimental data point, as well as controls, were assessed in triplicates. CC50 is defined as the amount of Propranolol and PND concentration required to disrupt the plasma membrane integrity of 50% of the cell population, as compared with solvent-treated cells.

Figures:

![Graph showing CC50 of Propranolol on MDA-MB-231 cells](image)

**Figure 3.1: Effects of Propranolol on cell viability of MDA-MB-231 cells to determine CC50.** The MDA-MB-231 cell line was subjected to distinct propranolol doses. Cell viability was measured for 24 hours to attain the half maximal effective concentration. Concentrations ranged from 15uM and increased in increments of 15uM to a final concentration of 105uM. At 24 hours the CC50 falls in between 30uM-45uM, where cell viability ranges between 44%-55%. A previous study done by our lab showed the CC50 value at 48 hours to be around 74uM, so we chose to use 37uM for the CC50 at 24 hours. Untreated MDA-MB-231 cells along with hydrogen peroxide and DMSO treatments were used as controls to help verify the accuracy of the experiment.
Figure 3.2: Propranolol in combination with PND show antagonistic effects on the MDA-MB-231 cell line. Propranolol was tested at different concentrations ranging from 10uM-60uM for the first 24 hours and PND treatment of 5uM for the last 24 hours. No synergistic affects were noted after 48hours. At 40uM, close to the CC50 of propranolol (37uM), only 16.4% of cell death is seen. Untreated MDA-MB-231 cells along with hydrogen peroxide and DMSO treatments were used as controls to help verify the accuracy of the experiment.

Figure 3.3: Effects of Propranolol on cell viability of MCF-7 cells to determine CC50. The MCF-7 cell line was treated with distinct propranolol doses ranging from 10uM-80uM. Cell viability was measured for 24hours to attain the half maximal effective concentration. Untreated MCF-7 cells along with hydrogen peroxide and DMSO treatments were used as controls to help verify the accuracy of the experiment. The control cells treated with DMSO have a higher-than-normal number of dead cells. The high amount of dead cells could be contributed to the DMSO therefore the findings of this experiment are invalid.
**Figure 3.4: Effects of Propranolol on cell viability of MCF-7 cells to determine CC50.** The MCF-7 cell line was subjected to a range of propranolol doses 10uM- 80uM. Cell viability was measured for 24 hours to attain the half maximal effective concentration. Untreated MCF-7 cells along with hydrogen peroxide and PBS treatments were used as controls to help verify the accuracy of the experiment. The PBS control is within a normal range for this experiment. At 24 hours the EC50 falls in between 20uM-40uM, where cell viability ranges between 45%-61%. The EC50 chosen for the experiment is 35uM.

**Figure 3.5: Propranolol in combination with PND show additive activity on cell viability in the MCF-7 cell line.** MCF-7 cells were treated with different doses of Propranolol ranging from 120uM to 10uM for 24 hours followed by the treatment of PND at the EC50 (1.6uM) for the last 24 hours. Cell death occurs at high amounts at concentrations 120uM to 80uM.
Figure 3.6: PND in combination with Propranolol has cytotoxic activity on cell viability in the MCF-7 cell line. MCF-7 cells were treated with different doses of PND ranging from 10uM for 24 hours followed by the treatment of Propranolol at the CC50 (35 uM) for the last 24 hours. High percentage of cell death occurs at 10uM-1uM and begins to decrease at the lowest dose of PND at .5uM.
Chapter 4: Testing Additional Breast Cancer Cell Lines HCC70 and MDA-MB-468 to try to Better Understand the Variability of Effects of Propranolol and PND Treatment

INTRODUCTION:

New and continuous research has highlighted different drug combinations that can lead to synergistic effects that can be effective for treating different types of cancers [131,135,139]. Although synergistic activity between drugs is often very rare, there are some promising combinations such as, CHK1 and WEE1 inhibitors, used against lung cancer [135]. Drug combinations are becoming more widely sought out for the fact that they can be used to overcome resistance, as well as enable better efficacy [135]. In vitro drug studies may not always translate to human trials, especially because cancer varies from patient to patient. A recent study suggests that discovering reagents that utilize a shared pathway or, even similar protein-protein interactions, will often tend to have more synergistic activity [135]. Because chemotherapy drugs alter cancer cells at different points of their cell cycles, using a combination of drugs can help increase the chance that more of the invasive cells will die. It has also been shown that if the drugs are targeting the cancer cells through two different mechanisms, such as disruption of DNA synthesis while the other is interfering with protein synthesis, this lessens the chance of cell survival, ultimately inhibiting cell proliferation as well as drug resistance [131,132,135].

Many cell lines have differing effects when treated with the same compounds, and sometimes the differences can be extreme, as seen with the MCF7 and MDA-231 cell lines. In this chapter we will focus on evaluating any possible synergistic effects with the MDA-468 and HCC70 breast cancer cell lines, while using the non-cancerous MCF10A cell line as a “normal” cell line control. Like the MDA-231 cell line, both the MDA-468 and HCC70 cell lines are TNBC. I
hypothesize that the results will differ but will show a reduction in cell viability. I believe this for the fact that the reagents could be targeting different mechanisms and/or receptors and molecular pathways in each cell line. Each cell line is unique, and the molecular morphology differs from cell to cell, which makes it hard to predict what the outcomes will be, but once the concentrations are determined further studies into the molecular mechanisms should be completed.

For the experiments carried out throughout this chapter the Pico Automated Cell Imaging System was used rather than the In-Cell Analyzer. This protocol uses the same set up and two-dye system to count live and dead cells, but unlike the In-Cell Analyzer, the Pico combines digital microscopy with automated brightfield fluorescence and digital confocal imaging to create high-resolution images and powerful analysis within minutes.

**Propranolol and PND Prove to be Ineffective on the Normal Breast Epithelial MCF10-A Cells**

MCF10-A cells were isolated from the healthy mammary gland of a 36-year-old female patient, and this cell line is one of the most used models to evaluate the efficacy of cancer treatments. The CC50 for PND was determined in an earlier study in the Villanueva paper to be around 6.6uM while the CC50 for Propranolol seems to be greater than 100uM ([Figure 4.1](#))[134]. To determine if the concentrations used throughout Chapter 3 have any impact on the non-malignant cell line, the same concentration of Propranolol (100uM-10uM) and PND (1.6uM) were used ([Figure 4.2](#)). After 48hrs the Pico System was used, the results showed that the cell line seems to be relatively unaffected by the drug combination. At the highest dose combination (100uM Propranolol + 1.6uM PND) only about 10% of cell death is seen. As the dosages lower cell viability starts to increase and reaches almost 95%. The data suggests that propranolol and
PND treatment have promising outcomes because most malignant cell lines show a dose dependent viability while normal cells remain unphased after the treatment period. Previously established CC50’s are presented to show accurate results.

**Propranolol and PND Induce Possible Apoptotic Activity in the HCC70 Cell Line**

HCC70 cells were isolated from ductal carcinoma from a 49-year-old female patient and is classified as a TNBC. The HCC70 cells were subjected to different concentrations of propranolol ranging from (100uM -10uM) for the first 24 hours followed by PND treatment at the CC50. According to the Villanueva paper, the CC50 for this cell line was determined to be 1.7uM [134]. In chapter 1 it was also determined that the CC50 for propranolol was around 20uM (Table 1.4).

The results indicate at the highest concentration of propranolol (100uM) + PND almost 95% of cell death can be seen (Figure4.3). Cell viability is still very low at treatment with 80uM propranolol + PND, but starts to increase around the treatment of 40uM propranolol +PND. At the lowest dose of propranolol (10uM) + PND cell viability is only around 60%. This data suggests that propranolol in combination with PND does not have synergistic activity but rather additive activity. The treatment at (10uM of propranolol + 1.5uM of PND), which is the CC50 of propranolol, seems to be close to 50% of the cytotoxic concentration.

**Propranolol and PND Work Together to Reduce Cell Proliferation of MDA-MB-468 Cells**

MDA-MB-468 cells were isolated from a 51-year-old female patient with metastatic breast adenocarcinoma and is also classified as a TNBC. The CC50 values were determined in earlier experiments and showed PND to be 1.7uM and propranolol around 68uM (illustrated on figure 4.4). The cells were treated with different doses of propranolol ranging from 100uM-10uM for the first 24hrs, followed by the treatment with 1.7uM PND for the last 24hrs. The results show that at the highest concentration of 100uM propranolol + PND cell viability is around 60%, and as the dosages lower cell viability starts to increase and reaches almost 70% at the treatment of 10uM.
propranolol + PND. The data reveals the ideal dose to induce 50% cytotoxicity would be > 100uM propranolol + PND. The combination induced additive effects in the MDA-MB-468 cell line.

As the dosage begin to lower, it is noted that cell viability is somewhat affected with only about 30% cell death occurring.

**DISCUSSION:**

The results from this chapter demonstrated that different effects can be seen from cell line to cell line. The MCF10-A cells showed that they were relatively unaffected by the different treatments of propranolol + PND, while the dose combination showed an additive effect in the HCC70 and MDA-MB-468 cell line. At the highest concentration (100uM propranolol +PND) about 20% cell death is observed. The cell viability of the HCC70 cells was affected at concentrations starting as low as 20uM propranolol + PND, where about 60% of cell death is seen. Cell viability of the MDA-MB-468 cells remained in a constant range throughout the different dosage treatments, where cell death was observed between about 45%-30%. The cell viability of the MDA-MB-468 cells wasn’t affected as drastically as the HCC70 cells. Overall, the cells seem to show a cell viability decrease response to the different drug combinations and could ultimately be leading these cells into apoptosis. More experiments should be done on a broad spectrum of breast cancer cell lines to verify these results.

**MATERIALS AND METHODS:**

**Cell culture and treatment**

The MDA-MB-468 cell line was grown in DMEM medium (Hyclone) supplemented with 10% FBS (Hyclone) and 100 U/mL of penicillin and 100μg/mL of streptomycin (Lonza). HCC70 cells were grown in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL of penicillin and 100μg/mL of streptomycin (Lonza). MCF10-A cells were grown in DMEM F/12 media containing 10% FBS, 100 U/mL of penicillin and 100μg/mL of streptomycin. Furthermore,
20ng/mL of epidermal growth factor (EGF), 0.5μg/mL of hydrocortisone, and 10μg/mL of insulin was added to the MCF10-A cell line. All cells were maintained in a water-jacketed CO2 incubator at 37C. DL-propranolol hydrochloride (Acros Organics) and doxorubicin hydrochloride (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and were administered as indicated for each experiment. As a solvent control, DMSO and PBS were tested at the same concentration (1% v/v) as contained in the experimental samples. Also, untreated cells were used to establish the background of dead cells provoked by factors inherent to cell cultures and cell manipulation.

**Live/dead staining Differential nuclear staining assay to quantify cell death**

To analyze the potential cytotoxic activity of Propranolol and PND, the Differential Nuclear Staining (DNS) assay, live-cell bio-imaging was utilized. For this assay, cells were seeded at 10,000 cells/well density in a 96-well plate in 100μl of culture media, incubated overnight, and treated with a gradient of Propranolol for the first 24hrs and PND at 5uM for the last 24hrs for a total of 48hrs incubation. Hoechst 33342 and Propidium iodide, which are two fluorescent nucleic acid intercalators were added to each well two hours prior to analysis; at a final concentration of 1 μg/ml each. Due to its high permeability, Hoechst stains all the cells (total dead and alive), whereas PI only stains dead or dying cells. Montages of 2 by 2 images were captured directly from each individual well of the culture plates by using a multi-well plate reader Pico Automated Cell Imaging System. The following controls were included in every single plate: PBS and DMSO as solvent/vehicle control, Hydrogen peroxide as a positive control for cytotoxicity, and untreated cells to determine the background of toxicity due to cell manipulation and intrinsic factors usually associated with the culture protocol. Each experimental data point, as well as controls, were assessed in triplicates. CC<sub>50</sub> is defined as the amount of Propranolol and PND concentration required to disrupt the plasma membrane integrity of 50% of the cell population, as compared with solvent-treated cells.
Tables:

Table 4.1: Different Breast Cancer Cell Lines Used to Establish the Ideal Combination (CC50)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Most Ideal Combinations (CC50’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>&gt;60uM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>10uM Propranolol + 1.6uM PND</td>
</tr>
<tr>
<td>MDA-468</td>
<td>100uM Propranolol + 1.7uM PND</td>
</tr>
<tr>
<td>HCC70</td>
<td>15uM of Propranolol + 1.5uM of PND</td>
</tr>
<tr>
<td>MCF10-A</td>
<td>&gt;100uM</td>
</tr>
</tbody>
</table>

Figures:

Figure 4.1 Effects of Propranolol on cell viability of the MCF10-A cell line to determine CC50. The MCF10-A cell line was treated with different concentrations of Propranolol (100uM-10uM) for 24 hours to establish the CC50. It has been noted that this cell line may be sensitive to DMSO, and for that purpose to eliminate any bias PBS was used as the vehicle control. After 24 hours Propranolol induces a slight effect on the non-tumorigenic epithelial cell line. At the highest concentration (100uM) a slight reduction in cell viability is seen, but about 80% of cells remain healthy. As the concentrations lower there is little to no difference in cell viability.
Figure 4.2 Propranolol and PND show little to no effect on cell viability in the MCF10-A cell line. The MCF10-A cell line was treated with different concentrations of Propranolol (100uM-10uM) for the first 24 hours and then treated with a constant dose of PND at its CC50 (1.6uM) for the last 24 hours. After 48 hours, these non-cancerous breast cells show they are relatively resistant to propranolol and PND doses. The combination doses seem to have minimal effect on this cell line. The CC50 for PND was previously established to be 6.6uM, but this data reflects the 1.6uM used throughout the study in Chapter 3. Cells were treated at both CC50’s of PND (6.6uM and 1.6uM) to show the concentration required to disrupt the plasma membrane integrity of 50% of the cell population.

Figure 4.3 Propranolol and PND could potentially cause apoptosis in HCC70 cells. HCC70 cells were treated with different concentrations of Propranolol (100uM-10uM) for the first 24 hours and then treated with a constant dose of PND at its CC50 (1.5uM) for the last 24 hours. After 48 hours the data shows a dose dependent viability decrease. At higher concentrations (100uM and
80uM) almost 90-80% cell death occurs. As concentrations lower, cell viability begins to increase. The most effective concentrations seem to be 15uM of Propranolol + 1.5uM of PND. The CC50s for Propranolol and PND were previously established in an earlier publication and was determined to be 20uM and 1.5uM.

Figure 4.4 Propranolol and PND reduce cell viability of MDA-MB-468 cells. The MDA-MB-468 cell line was treated with different concentrations of Propranolol (100uM-10uM) for the first 24 hours and then treated with a constant dose of PND at its CC50 (1.7uM) for the last 24 hours. After 48 hours the data shows a trend towards a dose dependent viability decrease. At higher concentrations (100uM and 80uM) about 60% of the cells are alive, while about 40% are dead. As concentrations lower, cell viability begins to increase, but even at the lowest dosage of 10uM cell viability is still affected with about only 30% of cell death observed. The CC50 for Propranolol and PND was previously established in an earlier publication and was determined to be 68uM and 1.7uM.
CONCLUSION:

Collectively, all our data suggests that the incorporation of the β-AR antagonist propranolol may decrease tumor proliferation not only in localized breast tumors, as we previously demonstrated, but also in advanced stages of disease. β-ARs are expressed across many different cancer types and preclinical and clinical efficacy has been reported following inhibition of these receptors across a large number of diverse cancers \[61,140\]. Propranolol induced a plethora of effects including reducing the Ki67 proliferation index in a three-week window, not only in an early-stage breast cancer patient, but a late-stage breast cancer patient as well. The cascade of events led to the reduction of a handful of mitogenic signaling regulators and increased pro-apoptotic proteins. A reduction in the pro-survival protein Bcl2 led to an increase in a handful of caspases and ultimately triggered apoptosis. The propranolol-mediated, anti-cancer mechanisms reported in the current study using breast cancer models may translate into a much broader group of cancers, paving the way for an inexpensive and non-toxic drug to be incorporated as an adjuvant reagent to current treatment regimens. The findings in chapter 1 and 2 have been published in two journals, the Oncotarget Journal and the Biomedical Journal, and together have been cited over 100+ times in the hopes of shedding light into the value repurposing drug reagents holds.

While it may seem very logical to think that because reagents work alone, they will also have synergistic activity, we still lack and are limited to understanding the underlying cellular processes. Many cancers differ from patient to patient and therefore the way the cancer cell will respond to treatment will vary. It has been suggested that reagents that only have two targets do not have as much synergistic activity as reagents that have more than two targets. It has been suggested in the paper by Nair at el. that synergy is more likely to emerge from targeting a single
pathway or two interacting pathways, than by targeting two completely distinct pathways or functional modules of the cell [131]. It is important to note that the combination of propranolol and PND showed positive outcomes in terms of reducing cell viability on the breast cancer cell lines MCF-7, HCC70, and MDA-MB-468, while having relatively no effect on the MDA-MB-231 breast cancer cell line. The ABC transporter has been implicated in many studies as one of the major culprits that limit the efficacy of the drug reagents and it has been reported that many cancer cells that show multiple drug resistance often express multiple ABC transporters, causing an upregulation, as compared to non-tumorigenic cell [137,138]. In a recent publication it was shown that the MDA-MB-231 cell line has an upregulation in the expression of the ABC transporter and that could be one possibility as to why the differences were seen in this cell line versus the other three [141].

While we hypothesized, we would see synergistic activity throughout the cellular studies, none was noted and in one instance antagonistic effects were observed. All the cell lines, except for the MDA-MB-231 and MCF10-A cells, exhibited additive reactions. Gene mutational status in cancer cells also makes it hard to predict how potential chemotherapies react and thus can also play a major role in some sensitivity to synergism as compared to other models. Repositioning drugs for other therapeutic uses is a powerful desire and coupling that with synergistic activity with other, low side-effect reagents, could provide benefits by allowing lower drug doses than used with single agents and possibly more cost effective than current chemotherapy treatments.
References:


118. Grytli HH, Fagerland MW, Fossa SD, Tasken KA, Haheim LL. Use of beta-blockers is associated with prostate cancerspecific survival in prostate cancer patients on androgen deprivation therapy. Prostate 2013;73:250e60.


Vita

Alexa Montoya was born in El Paso, Texas. She attended elementary and middle school in the El Paso District and graduated from Chapin High School with honors in June 2010 while attending early college courses at the local community college. Once she attained her Associates degree she entered University of Texas at El Paso in 2013 and in May 2015 received the degree of Bachelor of Science in Molecular and Cellular Biochemistry. That following August she entered the Master of Science program and in 2017 received her first publication that was printed in the Oncotarget Journal titled “Use of Non-selective β-blockers is Associated with Decreased Tumor Proliferative Indices in Early-stage Breast Cancer”. In 2019 she transferred to the Doctoral program and received her second publication that was printed in the Biomedical Journal titled “The β-adrenergic Receptor Antagonist Propranolol Alters Mitogenic and Apoptotic Signaling in Late-Stage Breast Cancer”. During this time, she worked as a Teaching Assistant for multiple classes that include genetics and molecular cell biology. After gaining further knowledge into carcinogens, she opened a successful skin care business and continued her studies in chemical formulations. In 2023 she successfully completed her qualifying exams and dissertation defense and received her Doctor of Philosophy in Biosciences in December 2023.