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ENHANCING CHEMOTHERAPEUTIC EFFICACY OF DOXORUBICIN THROUGH COMBINATION THERAPY WITH RAPAMYCIN AND SILDENAFIL, WHILE PROVIDING CARDIOPROTECTION IN BREAST CANCER MODELS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

By

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This paper is dedicated to my loving immigrant grandparents (Chambo and Chach) who have sacrificed everything for their family. They instilled the value of education in me, and have taught me first-hand the importance of dedicating myself to science, medicine, and helping others.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning	Page
DOX	Doxorubicin	
Sild	Sildenafil	
Rapa	Rapamycin	
PDE5	Phosphodiesterase-5	
FDA	Food and Drug Administration	
NIH	National Institute of Health	
ErbB2	Human epidermal growth factor receptor 2 (formerly known as HER2)	
CVD	Cardiovascular Disease	
LVEF	Left ventricular ejection fraction	
LVFS	Left ventricular fractional shortening	
ROS	Reactive oxygen species	
TNBC	Triple-negative breast cancer	
ER	Estrogen Receptor	
PR	Progesterone Receptor	
PDE5I	Phosphodiesterase-5 Inhibitor	
cGMP	Cyclic guanosine monophosphate	
PKG	Protein Kinase G	
sGC	Soluble guanylate cyclase	
NO	Nitric oxide	
eNOS	Endothelial nitric oxide synthase	
Bcl-xL	B-cell lymphoma-extra large	
FAP-1	Fas-associated phosphatase-1	
I/R injury	Ischemia/Reperfusion injury	

APD	Action Potential Duration
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
S6K	p70s6 Kinase
PI3K	Phosphatidylinositol 2-kinase
Ras Rheb-GTP	Homolog enriched in brain GTPase
ULK1/2-ATG	Unc-51-like kinases 1 and 2
Trast	Trastuzumab
LVD	Left ventricular dysfunction
AFA	Afatinib
TB	Trypan Blue
PBS	Protein Buffer System
FBS	Fetal Bovine Serum
PI	Propidium Iodide
PARP	Poly (ADP-ribose) polymerase
ECG	Electrocardiography
LVIDd	Left ventricular end-diastolic diameter
LVIDs	Left ventricular end-systolic diameter
PWDT (LVPWd)	Left ventricular end-diastolic posterior wall thickness
PWST (LVPWs)	Left ventricular end-systolic posterior wall thickness
AWDT (LVAWd)	Left ventricular end-diastolic anterior wall thickness
AWST (LVAWs	Left ventricular end-systolic anterior wall thickness
SV	Stroke Volume
CO	Cardiac Output
HR	Heart Rate

ABSTRACT

Background:

Doxorubicin (DOX) is a widely used chemotherapeutic agent for various cancers, including breast cancer, but its effectiveness is limited by serious multi-organ dysfunction, such as cardiotoxicity causing heart failure, myocardial ischemia, arrhythmias, and cardiomyopathy. It is crucial to prevent DOX-induced heart damage without compromising its cancer-fighting abilities. Sildenafil (Sild) and Rapamycin (Rapa), which respectively inhibit phosphodiesterase-5 (PDE5) and mTOR, have shown promise in enhancing DOX's anticancer effects, while mitigating its cardiotoxicity. This study focuses on combining Sild and Rapa to enhance DOX's anticancer properties, while protecting the heart.

Methods and Results: Triple negative human breast cancer cells (MDA-MB-231) and murine breast cancer cells (4T1) were exposed to DOX (1 μM) with/without Rapa (100 nM), Sild (10 μM) for 24-72 hours. Cell death, cell viability assays and protein expression were examined after treatment. Spheroid culture of human epidermal growth factor receptor 2 (ErbB2; formerly known as HER2)-positive breast cancer cells (SKBr3) were treated with DOX (1 μM) and/or AG825 (ErbB2 inhibitor, 5μM) with/without Rapa (100 nM) and/or Sild (10 μM) for 48 hours, then medium was replaced with fresh medium so cells could further incubate for 48 hours. The medium from SKBr3 cells was then collected and used to treat human cardiomyocytes (AC16) for 48 hours to assess cardiomyocyte cell death and understand the cardioprotective properties of the drug combination. Combination treatment with Sild and Rapa significantly enhanced DOX-induced killing of all three-breast cancer cell lines but reduced DOX-induced cell death in AC16 cardiomyocytes, compared to individual drug treatment. Combination treatment notably suppressed mTOR signaling and the expression of Bcl2 (anti-apoptotic protein) but increased cleaved PARP (an apoptosis marker) in cancer cells. Additionally, the cardioprotective effect of the combination therapy was assessed in a murine *in vivo* model. Adult female C57/B6 mice were consecutively treated with DOX (6 mg/kg/twice a week; i.p. 2 weeks) and Afatinib (ErbB2 antibody, 10 mg/kg, oral gavage, 5 days/week, 2 weeks) either alone or in combination with Rapa (0.25 mg/kg/d, i.p.) and/or Sild (1.4 mg/kg/day; in drinking water) for 12 weeks. Combination therapy with Sild and Rapa alleviated DOX+Afatinib-induced cardiac dysfunction (Ejection Fraction and Fractional Shortening, assessed by echocardiography) with improved survival and body weight.

Conclusion: This study manifests a unique therapeutic strategy for breast cancer, chemo and immunotherapy in conjunction with Sild and Rapa, which augments the efficacy of cancer therapy, while safeguarding cardiac function. This research represents a promising advancement in promoting the health and longevity of breast cancer patients subjected to chemo-immunotherapy.

INTRODUCTION

Background

Cancer remains a formidable global health challenge resulting in morbidity and mortality worldwide. Over the past decade, despite a decline in age-adjusted incidence rates, the number of cancer survivors continues to rise due to an aging population, improved early detection, and advancements in treatment modalities (**Cherukuri et al, 2022**). Surgical interventions along with therapies like radiation, hormonal treatments, and chemotherapy, constitute the primary fight against cancer (**Cherukuri et al, 2022**).

Among all cancer types, breast cancer stands as the most diagnosed malignancy globally, surpassing lung cancer. In 2020, over 2.3 million new breast cancer cases were reported, resulting in 685,000 deaths globally (**Arnold et al, 2022**; **Sung et al, 2021)**. On the same scale, the burden of breast cancer is projected to exceed 3 million new cases annually by 2040, due solely to population growth and aging; underscoring the need for continued research and improved therapeutic strategies (**Arnold et al, 2022**). Age remains a predominant risk factor for breast cancer, with incidence rates peaking in older age groups, although younger populations in developing countries are increasingly affected due to evolving lifestyles and healthcare access; ~1.5% increase at age 40, ~3% at age 50, and ~4% at age 70 (**Miller et al, 2016; Cherukuri et al, 2022)**. Genetic predispositions, reproductive factors, and lifestyle choices are few among influences of breast cancer risks (**Wilkinson, 2022)**. Treatment of breast cancer typically involves a multimodal approach tailored to disease stage and the individual patient. Chemotherapy, a cornerstone in systemic treatment, includes anthracyclines like doxorubicin, renowned for their efficacy. While there are efficient cancer cytotoxic chemotherapies for breast cancer, other complications seem to arise from these treatments. Among a study of 63,566 female breast cancer patients, 15.9% experienced cardiovascular disease (CVD) as their main cause of death, with cancer-related deaths declining as women aged and as breast cancer progressed, while deaths from CVD rose (**Cherukuri et al, 2022**). Despite treatment advances, CVD has emerged as a notable concern among breast cancer patients and survivors.

Chemotherapy with Doxorubicin

Doxorubicin (DOX) is a chemotherapeutic agent derived from *Streptomyces peucetius* var *caesius* in the 1960's and was approved by the Food and Drug Administration (FDA) in 1974 to treat a variety of cancers, including breast, leukemia, thyroid, ovarian, multiple myeloma, gastric, sarcoma, and non-Hodgkin's and Hodgkin's lymphoma (**Johnson-Arbor & Dubey, 2023**). DOX is employed as a chemotherapy drug and has three major aspects causing its anti-neoplastic activity and toxicity: (1) bind to DNA causing intercalation and torsional stress to inhibit topoisomerase II, disrupting DNA replication and repair in rapidly dividing cells; (2) bind to cellular membranes to alter ion channels and fluidity; and (3) redox reaction with its semiquinone free radical generating reactive oxidative species (**Johnson-Arbor & Dubey, 2023**).

Administered intravenously typically at three-week intervals, doxorubicin distributes widely into tissues, with an elimination half-life of up to 48 hrs (**Johnson-Arbor & Dubey, 2023**). DOX has a distinct reddish liquid appearance and should be stored in a cool, dark place before use (**Johnson-Arbor & Dubey, 2023**). While the utilization of DOX in cancer treatment has been transformative, its efficacy is variable, dependent on cancer type, stage, and individual patient characteristics. Statistics on DOX's success rates demonstrate its impact on cancer treatment outcomes. For instance, in breast cancer, DOX-containing regimens have shown response rates ranging from 40% to 80%, depending on the specific subtype and stage of the cancer (**Bao et al. 2011**). DOX has contributed to improved survival rates for other conditions including Hodgkin lymphoma, Ewing's sarcoma, acute lymphoblastic leukemia, etc. (**Johnson-Arbor & Dubey, 2023**).

Doxorubicin-induced Cardiotoxicities

Cardiovascular toxicity is the most important adverse effect of chemotherapy in cancer patients, leading to an increased risk of morbidity and mortality. As a chemotherapeutic agent, DOX's clinical utility is significantly tempered by cardiotoxicity; the harmful effects that can directly or indirectly impact the heart as the maximum cumulative dose approaches 550 mg/m2 (**Johnson-Arbor & Dubey, 2023**). Directly DOX may cause structural damage to the heart muscle, while indirectly, it can lead to conditions like thrombosis and dynamic blood flow changes. According to the Cardiac Review and Evaluation Committee, cardiotoxicity in patients receiving anticancer therapies is characterized by several conditions: decreased left ventricular

ejection fraction (LVEF), tachycardia, symptoms of heart failure, and reduction in LVEF to <55% with heart failure signs; all of which manifest as acute or chronic complications (**Gabani et al, 2021; Kong et al, 2022).** Acute cardiac toxicity includes reversible conditions such as myopericarditis and arrhythmias, affecting up to 26% of treated parents. Chronic toxicity, characterized by irreversible cardiomyopathy and congestive heart failure, poses a significant long-term risk, with incidence rates around 1.7% that may develop months to years after therapy (**Kong et al, 2022**). Risk factors for DOX-induced cardiotoxicity include cumulative drug, patient age extremes, combinant cardiotoxic therapies, and prior radiation exposure (**Kong et al, 2022**). A longitudinal cohort study of 277 breast cancer patients undergoing DOX treatment indicated a persistent 4% decline in LVEF three years after the start of treatment, emphasizing the need for vigilant cardiac monitoring during and after chemotherapy (**Cherukuri et al, 2022**). Addressing the cardiotoxic challenges of DOX requires interdisciplinary efforts to carefully monitor during treatment, and personalize treatment approaches (**Rivera, 2003; Fisher et al. 2005; Das et al. 2010**). Its cardiotoxicity is known in cardio-oncology to be caused by oxidative stress, DNA and mitochondrial damage, and cell death; ultimately, the cardiotoxic effects result in myocardial injury and left ventricular (LV) dysfunction (**Robinson et al. 2020**). Chemotherapy-induced cardiotoxicity potentiates several cardiac complications appearing immediately after treatment and even years following therapy and has therefore raised concern within oncology and cardiology.

DOX induces oxidative stress, the primary driver of cardiotoxicity, characterized by an imbalance in reactive oxygen species (ROS), resulting in damaged cellular structures and cell death. DOX undergoes one-electron reduction to form semiquinone, facilitated by NADPH oxidases and uncoupled nitric oxide synthases. This process leads to the production of superoxide and generates various ROS such as hydrogen peroxide, hydroxyl radical, and hydroxyl peroxide, triggering oxidative stress and ultimately programmed cell death (**Kong et al, 2022**). DOX's anti-tumor and cardiotoxic effects continue to evoke an immense interest in scientific research. This chemo drug has played a prominent role in cancer treatment- 32% of breast cancer patients, 57 to 70% of elderly lymphoma patients, and 50 to 60% of childhood cancer survivors **(McGowan et al. 2017)**. Consequently, its resulting long-term cancer survivorship causes these patients to remain at risk of cardiac complications and mortality due to the adverse effects of DOX chemotherapy (**Kong et al, 2022**). Although breast cancer is of a

lower percentage of the statistics mentioned above, there is sufficient research showing DOX's efficacy in treating aggressive forms of breast cancer, such as triple-negative breast cancer (TNBC, absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (ErbB2), which often has limited treatment options due to its lack of hormone receptors and ErbB2 overexpression. Thus, combination therapies have been shown to inhibit breast tumor growth and have encouraged trials in TNBC patients (**Pal et al. 2010**).

Ongoing research aims to optimize the efficacy of DOX as a chemotherapy drug and investigate cardioprotective medications that mitigate DOX-induced heart damage, all while suppressing cancer progression. These efforts are geared towards improving survival rates among breast cancer patients. Overall, the prominence of DOX research in breast cancer reflects its pivotal role in current treatment strategies and ongoing efforts to refine and improve survival of all cancer patients. As of now, dexrazoxane is the sole FDA-approved medication for anthracycline-induced heart failure (**Bansal et al. 2019**). However, due to its myelosuppression, dexrazoxane may result in secondary malignant tumors (**Tahover et al. 2017**). Therefore, it is essential to research safer agents that treat anthracycline-induced heart failure.

The consequential cardiotoxicity from chemotherapy, radiation therapy, targeted therapies, and immunotherapies require integrated approaches from cardiologists and oncologists to manage breast cancer patients and improve their prognosis with holistic care.

Phosphodiesterase-5 Pathway

A promising therapeutic target is Phosphodiesterase-5 (PDE5), often overexpressed in several human cancers, notably breast cancer (**Catalano et al. 2019**). PDE5 inhibitors (PDE5I) like Sildenafil (Sild) have been extensively studied for their dual role in cardioprotective and cancer treatment (**Das et al. 2005, Fisher et al. 2005; Das et al. 2010**). These inhibitors prevent the degradation of cyclic guanosine monophosphate (cGMP), leading to increased levels of cGMP that activate protein kinase G (PKG) and subsequently open mitochondrial K_{ATP} channels, promoting smooth muscle vasodilation, as shown in **Figure 1** (**Fisher et al. 2005**). The interaction between DOX, sGC (soluble guanylate cyclase), and PDE enzymes modulates cGMP levels, suggesting a potential therapeutic role for the NO (nitric oxide)-sGC-cGMP signaling pathway in managing DOX-induced cardiotoxicity (**Catalano et al. 2019**). Additionally, the activation of mitochondrial K_{ATP} channels plays a crucial role in providing cardioprotection

against pharmacological treatments by a decrease in tissue ATP levels to inhibit apoptosis (**Akao et al. 2001; O'Rourke et al. 2000**). As shown in **Figure 2**, DOX generates ROS, which inhibits the ability of endothelial nitric oxide synthase (eNOS) and impairs blood vessel dilation, contributing to cardiotoxicity (**Catalano et al. 2019**). eNOS generates nitric oxide which stimulates guanylyl cyclase. This increases signaling molecule cGMP in smooth muscle cells which is catalyzed as inactive GMP by PDE5. This catalyzation decreases cGMP levels, decreasing protein kinase G (PKG), increasing transcriptional beta-catenin, allowing for increased cancer cell proliferation (**Catalano et al. 2019**). PDE5I blocks the catalysis of cGMP to GMP, thus increasing cGMP levels, increasing PKG, lowering beta-catenin, and lowering cancer cell proliferation (**Catalano et al. 2019**). The PDE5 mechanism highlights the potential for developing novel therapeutic strategies aimed to target cancer cells and enhance cardioprotection.

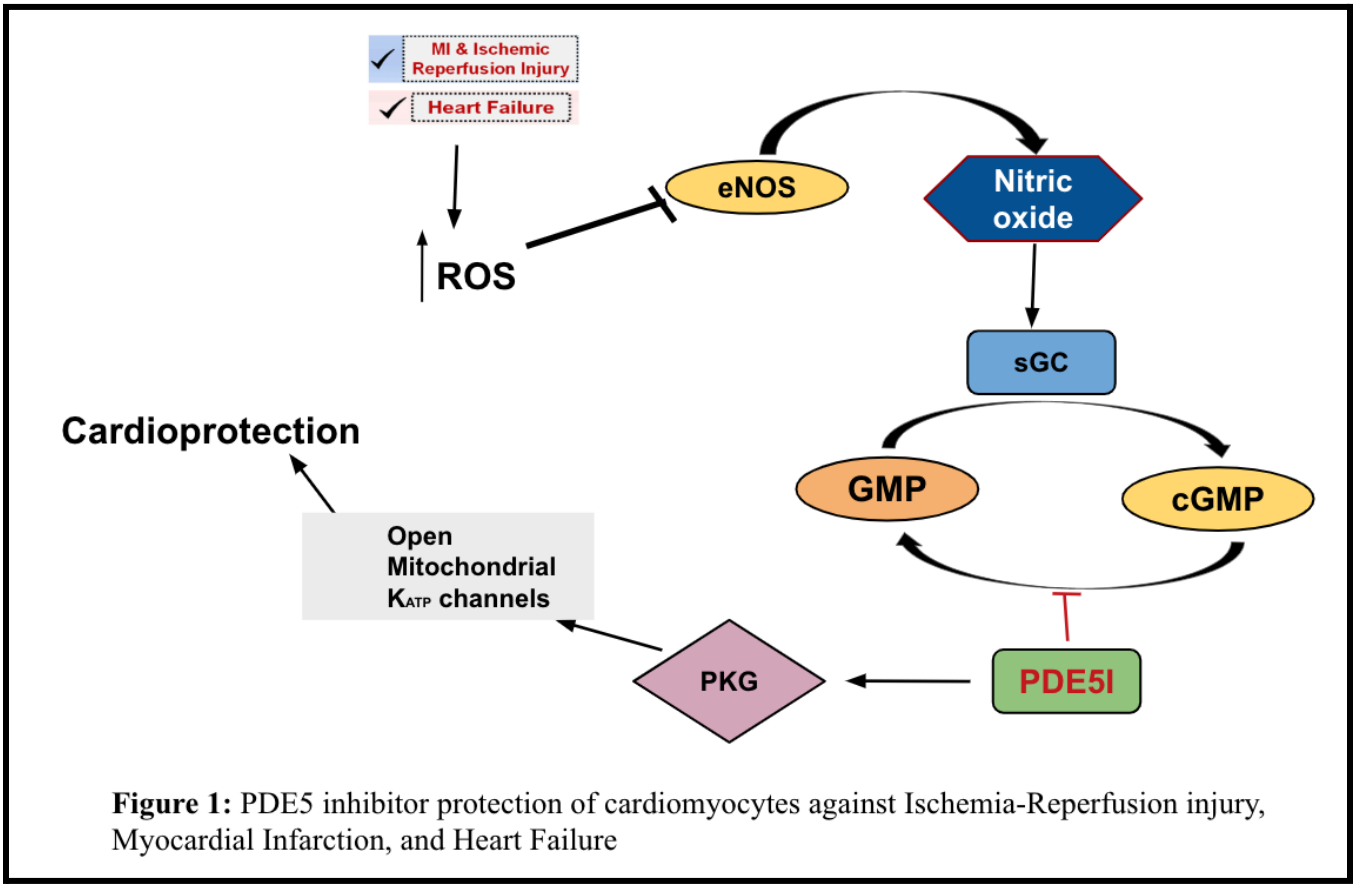

Figure 1: PDE5 inhibitor protection of cardiomyocytes against Ischemia-Reperfusion injury, Myocardial Infarction, and Heart Failure

PDE-5 has been overexpressed in different cancers, including lung, colon, metastatic breast cancers, and bladder squamous carcinoma (**Catalano et al. 2016; Karami-Tehrani et al.**

2012; H.N. Tinsley et al. 2010). Originally developed for erectile dysfunction and pulmonary hypertension, Sild has demonstrated the ability to enhance cancer cell apoptosis by suppressing PDE5, which could activate the cGMP-PKG pathway to mediate many signaling pathways associated with cellular apoptosis or growth suppression (cell cycle arrest) (**Piazza et al. 2020**). Sild enhances the cytotoxic effect of chemotherapeutic agents in tumor cells by downregulating anti apoptotic proteins, B-cell lymphoma-extra large (Bcl-xL) and Fas-associated phosphatase-1 (FAP-1) expression and enhancing ROS generation, phosphorylating BAD and Bcl-2 with inducing caspase-3,8,9 activities and cell cycle arrest at G0/G1 phase (**Das et al. 2010, 2016; Haider et al. 2021**). These mechanisms not only potentiate the cytotoxic effects of DOX on cancer cells, but also enhance other chemotherapeutic agents like Docetaxel, while mitigating their associated side effects in clinical setting (**Das et al. 2010, 2016**; **Muniyan et al. 2020**).

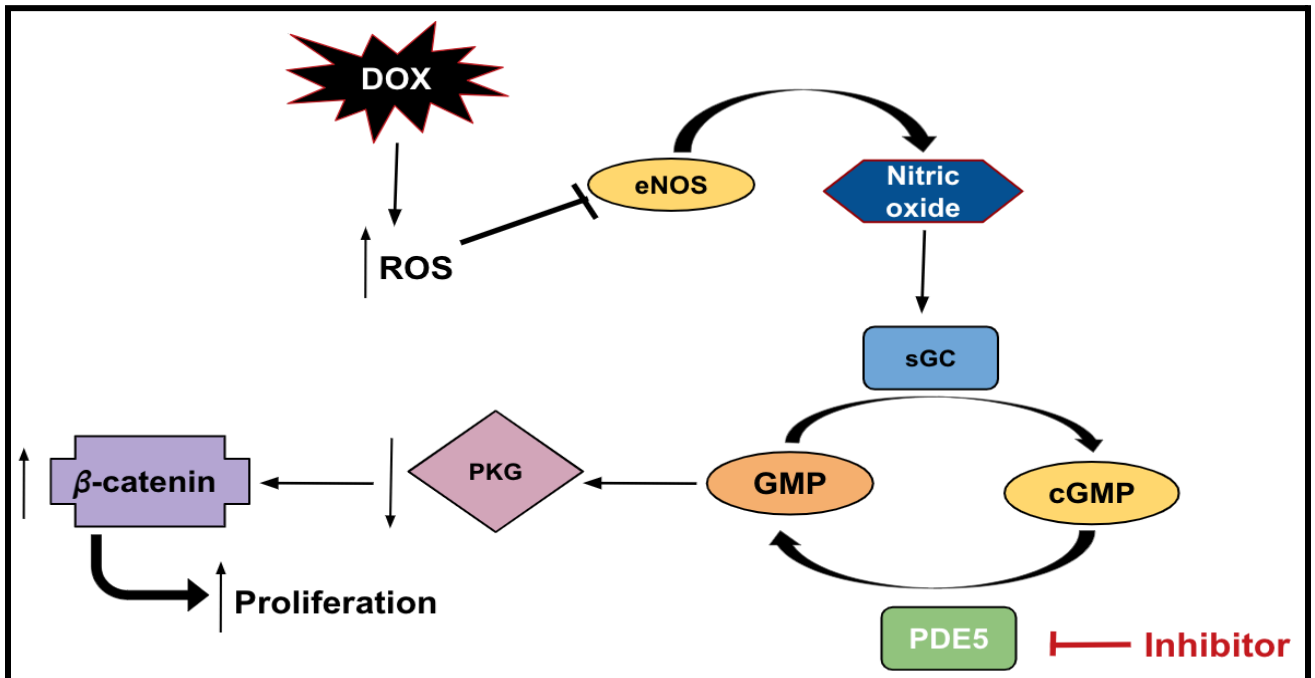

Figure 2: Phosphodiesterase- 5 pathway overexpressed in cancer cells and the consequential effect of Doxorubicin and PDE5 Inhibitor for cancer cell cytotoxicity.

Moreover, studies using animal models of ischemia/reperfusion (IR) injury have consistently shown reduced infarct size and improved post-ischemic functional recovery, underscoring the importance of apoptosis regulation (**Garlid et al. 1997**). Our laboratory showed that Sild reduces infarct size post IR injury and improves cardiac function following myocardial

infarction or DOX chemotherapy (**Das et al. 2005, 2010; Fisher et al. 2005**). In their investigation of Sild's protective effects against DOX-induced cardiotoxicity in mouse hearts, the Das Lab found that Sild reduces cardiomyocyte apoptosis and improves left ventricular function in chronic DOX models (**Fisher et al. 2005; Das et al. 2010**). Additionally, the study observed that in mice treated with DOX, the prolongation of the ST-interval is associated with an elongated action potential duration (APD) following the QRS complex. Action potential duration was also increased in Purkinje fibers after exposure to DOX. In isolated cardiomyocytes exposed to DOX, action potential duration notably increased due to ROS generated by DOX (**Fisher et al. 2005**). Consequently, mice treated with Sild alongside DOX exhibited consistent ST-intervals as that of control group throughout the course of the study, highlighting Sild's cardioprotective effects (**Fisher et al. 2005**). Additionally, administering DOX to nude mice bearing PC-3 (human prostate cancer) flank tumors resulted in a decrease in tumor volume. Concurrent administration of Sild further reduced tumor volume induced by DOX. Similarly, the ratio of tumor weight to body weight was decreased with Sild co-administration for the same mice (**Das et al. 2010**). Previous studies highlight the potential for Sild to reduce cancer cells while protecting cardiomyocytes.

mTOR Pathway

The mTOR pathway plays a pivotal role in regulating cellular processes by forming two distinct complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which are distributed across various intracellular organelles such as the nucleus, lysosome, mitochondria, and endoplasmic reticulum (ER) (**de la Cruz López et al. 2019)**. Both mTOR complexes are substantial in size, with mTORC1 composed of six known protein components to facilitate protein synthesis via p70s6 Kinase (S6K) phosphorylation, while mTORC2 consists of seven identified protein components to govern cell proliferation, metabolism, and survival by phosphorylating AKT at S473 (**Das et al. 2014; Laplante et al. 2012; Saxton & Sabatini, 2017)**. By utilizing the Ras/phosphatidylinositol 2-kinase (PI3K)/Akt/mTOR pathway, mTOR serves as a critical modulator that maintains the balance between cell growth and cell death in response to growth factors, nutritional conditions, and stress signals (**Laplante et al. 2012)**. Dysregulation of this intricate network is particularly significant in breast cancer, where mTOR overexpression can drive cell proliferation and survival mechanisms (**Laplante et al. 2012)**.

Rapamycin (Rapa), an mTOR inhibitor known for its immunosuppressive properties, is evaluated as a potential cardioprotective and anti-cancer agent based on mTOR's role as a crucial serine/threonine kinase that regulates cell proliferation and growth in various cancers, including breast cancer (**Ballou & Lin, 2008**). Inhibiting mTOR with Rapa targets both complexes, leading to cell cycle arrest and autophagy, thereby promoting programmed cell death (**Saxton & Sabatini, 2017**). mTOR's regulation extends to processes such as protein synthesis via the ribosomal S6 kinase pathway and modulation of autophagy, crucial in both cancer and cardiac diseases (**Wu et al., 2021**). The mTOR pathway shown in **Figure 3** plays a pivotal role in regulating cellular processes by integrating signals from growth factors, insulin, nutrients, and cytokines which bind to the human epidermal growth factor receptor (ERbB) family, go trigger the PI3K pathway and subsequently activate mTORC2. mTORC2 phosphorylates AKT, promoting cell survival, growth, and metabolism and inhibiting apoptosis (**Sciarretta et al. 2014**). Moreover, AKT suppresses the TSC1/2 complex, resulting in the activation of Ras homolog enriched in brain GTPase (Rheb-GTP). Subsequently, Rheb-GTP activates mTORC1, which phosphorylates S6K1 to promote protein synthesis and cell growth, or inhibits Unc-51-like kinases 1 and 2 (ULK1/2-ATG) to dampen autophagy, contingent upon cellular conditions (**Sciarretta et al. 2014**). In cancer cells, dysregulated mTOR signaling enhances cell

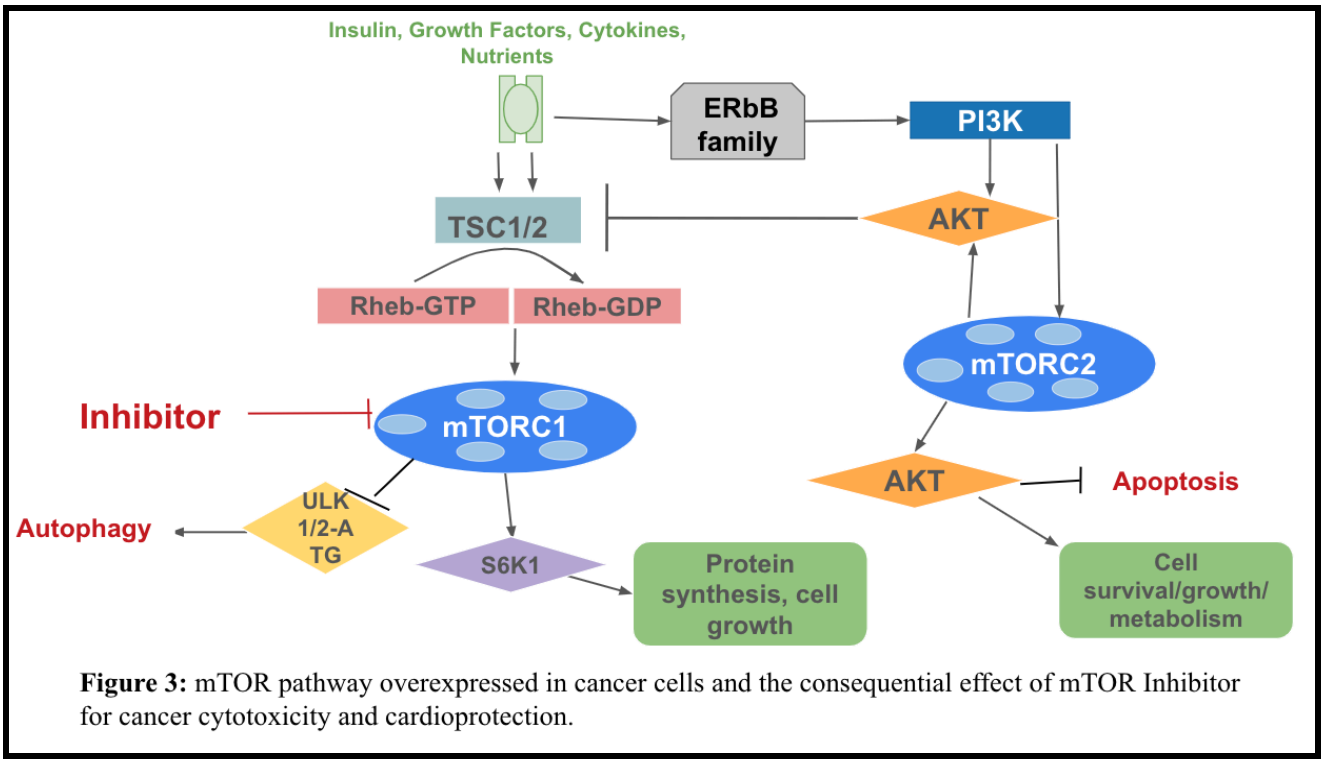

Figure 3: mTOR pathway overexpressed in cancer cells and the consequential effect of mTOR Inhibitor for cancer cytotoxicity and cardioprotection.

proliferation and survival, making it a target for therapeutic intervention (**Sciarretta et al. 2014**). Rapa inhibits mTORC1, thereby disrupting S6 phosphorylation, crucial for protein synthesis and growth signals for cancer cell proliferation. In contrast, in cardiac tissue, mTOR activation via the AKT/S6 pathway can protect against stress-induced damage by enhancing cell survival and function, highlighting the dual role of mTOR in cancer cells and cardioprotection (**Sciarretta et al. 2014**).

Moreover, Rapamycin's reported ability to reduce infarct size post IR injury and improve cardiac function underscores its potential as a cardioprotective agent in both normal and diabetic hearts (**Khan et al. 2006; Das et al., 2012, 2014**). A study showed a notable decrease in Bax expression (pro-apoptotic protein) in diabetic rabbit heart compared to DMSO-treated controls after Rapa treatment during reperfusion, accompanied by an increase in Bcl-2 expression (anti-apoptotic protein) (**Samidurai et al. 2020**). This resulted in an elevated Bcl-2 to Bax ratio with Rapa treatment compared to the DMSO group (**Samidurai et al. 2020**). Similarly, following four weeks of Rapa treatment, cardiac function was evaluated in C57 and diabetic mice using echocardiography (**Das et al. 2014**). Compared to the nondiabetic C57 control mice, db/db mice showed significant impairment in cardiac, evidenced by reduced fractional shortening (FS) and ejection fraction (EF) (**Das et al. 2014**). In contrast, Rapa treatment improved cardiac function in db/db mice, as indicated by increased fractional shortening and ejection fraction (**Das et al. 2014**). While there were no changes in left ventricular end-diastolic diameter between groups, left ventricular end-systolic diameters were significantly higher in db/db mice compared to C57 control and Rapa-treated db/db mice (**Das et al. 2014**). Rapa's multifaceted effects through mTOR inhibition not only hold promise in cancer therapy by suppressing mTORC1-driven cell growth but also in cardioprotection by modulating pathways like JAK2-STAT3 and enhancing the Bcl-2 to Bax ratio to mitigate ischemic/reperfusion injury (**Das et al., 2012**). In precondition against myocardial I/R injury, Rapa treatment group resulted in a lower infarct size and cardiomyocyte apoptosis of adult male ICR mice compared to DMSO. Consequently, the Rapa group increased left ventricular EF compared to the DMSO group (**Das et al., 2012; Khan et al. 2006**). Similarly, Rapa treatment at reperfusion reduced infarct size of adult male C57 mice hearts, compared to DMSO (**Filippone et al. 2017**). While the I/R + DMSO group notably decreased FS, I/R+Rapa showed improved FS. Likewise, the post-I/R Bax expression was significantly reduced in the cardiomyocytes and Bcl-2 increased following Rapa

treatment as compared with DMSO. The study showed an increased Bcl-2 to Bax ratio of I/R with Rapa treatment compared to I/R+DMSO (**Filippone et al. 2017**). The intricate balance of mTOR signaling in cancer cells versus cardiac tissue underscores its potential as a therapeutic target not only for cancer treatment but also for cardioprotection in clinical settings.

Immunotherapy

Human epidermal growth factor receptor 2 positive (ErbB2+) breast cancer is associated with more invasive tumors, accounting for nearly 25% of all breast cancer cases (**Liao et al. 2016**). It is frequently managed using ErbB2 receptor-targeted therapies, such as Trastuzumab (Trast, a kinase inhibitor), a monoclonal antibody directed against ErbB2 receptor, in combination with DOX. Trast works to downregulate the ErbB2 receptor and prevent cell proliferation and DOX amplifies this effect (**Lima et al. 2022)**.

However, a more severe and aggressive type of cardiac dysfunction has recently emerged in association with the concurrent use of Trast along with DOX. Trast was the initial FDA-approved kinase inhibitor designed for targeting ErbB2 overexpression in breast cancer treatment (**Yang et al. 2021**). Clinical investigations have demonstrated additive effects in controlling cancer proliferation and increasing survival rates among breast cancer patients receiving DOX and Trast, compared to each agent administered independently (**Liao et al. 2016**)

Consequently, there is evidence that Trast increases ROS production, activates caspase 9 and 3, induces apoptosis, and downregulates cardiomyocyte survival resulting in a notably high incidence of Trast-induced left ventricular dysfunction (LVD) which can lead to heart failure (16% with DOX+Trast vs. 3% with DOX alone) (**Yoon et al. 2019**). Thus, the simultaneous usage of DOX and Trast has been discontinued (**Das et al. 2022; Kuramochi et al. 2006**; **Yoon et al. 2019**). In a cohort study of 105 breast cancer patients treated with anthracycline chemotherapy alone or in combination with Trast, ECG assessments were conducted one and four years post-treatment initiation. The study revealed a higher incidence of myocardial damage after four years (6%) following initial anthracycline treatment (**Gabani et al. 2021**). Furthermore, the combination of anthracycline and Trast exacerbated damage compared to anthracyclines alone, leading to increased rates of cardiomyopathy, diastolic dysfunction, and a greater decline in LVEF. Importantly, diastolic dysfunction preceded or was associated with all

cardiomyopathy cases (**Gabani et al. 2021**). Therefore, more research is needed to understand if the dysfunction may be an early indicator of cardiomyopathy development.

Thus, it is imperative to develop novel therapeutics that mitigate DOX-induced cardiotoxicity, with or without ErbB2 inhibitor co-administered, while maintaining the anti-tumor effect of the combined regimen. Although Trast is a widely studied drug for breast cancer therapy, it is commonly accepted in science that Trast may not bind with mouse ERbB2. Differences in amino acids alter Trast-ErbB2 binding with mouse ErbB2 (**Lewis et al. 2022**). Alternatively, there is a significant amount of research that supports the use of Afatinib (AFA, another irreversible ErbB family inhibitor) in murine cancer research. It is currently being studied in several trials and research projects as a single drug and in combination with other chemotherapy agents for tumor progression and *in vivo* growth (**Modjtahedi et al. 2014**). Further research and clinical trials are essential to understand the full spectrum of benefits and potential adverse effects of this treatment combination.

Therapeutic significance of combination treatment

Based on this background, we contemplate that this unique strategy of combination therapy with Sildenafil and Rapamycin may have powerful cardioprotective effects and amplified anti-cancer benefits based on the phosphodiesterase-5 and mTOR pathways. Sild and Rapa may emerge as unique chemotherapeutic enhancers when used in conjunction with DOX, aiming to improve the efficacy of breast cancer treatment and increase cardioprotection. Importantly, the cardioprotective qualities of this combination are crucial, as DOX is associated with adverse cardiac effects (**Fisher et al. 2005; Das et al, 2010**). The collective use of Sild, Rapa, and DOX may present a multifaceted approach that not only targets cancer cells but also safeguards the heart, thereby improving the safety and efficacy of chemotherapy.

The proposed approach of novel combination treatment (with DOX and/or Sild and/or Rapa) has promising and significant value for the future of oncology and cardiology. This study holds great significance because: 1) PDE5/mTOR inhibitor mechanisms in cardiomyocytes are likely to be conserved for cells damaged by chemotherapy drugs; 2) These drugs could be applicable to breast cancer patients of all ages treated with chemotherapy (DOX), as Sild and Rapa are both FDA approved drugs, which are reputable for use in adults and children; 3) Combination of chemo (DOX) and immunotherapies (Afatinib) causes severe cardiotoxicity in

cancer patients, which could be attenuated by Sild and Rapa co-treatment. Therefore, the outcome from this study could have novel therapeutic impact in the breast cancer patients treated with DOX with/without ERBB2 inhibitor.

OBJECTIVES

The following objectives are addressed to test DOX cancer cell cytotoxicity and DOX cardiotoxicity through combination therapy of DOX, Sild, and Rapa: (1) To determine the in vitro therapeutic potential of PDE5 and mTOR inhibition (Sild and Rapa respectively) in combination with the anticancer effect of DOX in TNBC cells. (2) To examine the effects of PDE5 and mTOR inhibition (Sild and Rapa respectively) on anticancer efficacy of DOX and ErbB2 inhibitor (AG825) in ErbB2-positive breast cancer cells, while protecting cardiomyocytes (AC-16). (3) To utilize a murine model to test the cardiac impact of combination therapy of DOX and Afatinib (AFA, ErbB2 inhibitor) with/without Sild and Rapa. This study aims to optimize DOX in combination with cardioprotective agents, Sild and Rapa, to enhance breast cancer treatment efficacy. Our hypothesis proposes that the combined treatment of DOX, Rapa, and Sild will induce greater cytotoxicity in tumor cells, while providing cardioprotective benefits compared to treatment with DOX alone.

RESEARCH STRATEGIES

Methods

Cell Death Assay: After 24 or 48 hrs (dependent on cell line) of treatment, cell death will be assessed on collected cells by Trypan Blue (TB) exclusion assay to display live (white) and dead (blue) cytoplasm, observed through optical microscopy. A cell suspension with 0.4% TB and protein buffer system (PBS) was obtained ensuring an appropriate ratio of cells. A microvolume of the sample was used on a hemocytometer to manually count stained and unstained cells, to ultimately create a percentage of cell death for each treatment group.

Flow Cytometry: Cell death and apoptosis will also be quantified by Alexa Fluor 488 Annexin V and Propidium iodide (PI) staining using flow cytometer for all treatment groups. Propidium iodide, an impermeable nucleic acid dye, stains to detect non-viable cells using flow cytometry.

Annexin V and PI negative indicate viable cells. Annexin V positive and PI negative staining are early apoptotic cells. Annexin V and PI positive cells display late apoptosis of cells in necrosis. Annexin V negative and PI positive indicate late apoptosis or necrotic cells.

Cell Viability Assay: Cell viability will be examined using MTS assay using Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Approximately, 1,000 breast cancer cells/well were plated with 100µl RPMI or DMEM media containing 10% Fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) into three 96-well plates for 24 hrs. Each row of the plate was subjected to a different treatment group depending on the cell line for 48 hrs with fresh media. Cell viability was measured using a different treated plate every 24 hrs until 72 hrs of treatment. Following plate aspiration, CellTiter reagent and media was added to each well with a ratio of 1:10 respectively and incubated for one-hour before recording the absorbance at 490 nm. For each treatment group, the average absorbance of the medium background was subtracted from all experimental well values to determine the absorbance proportional to the number of viable cells.

Quantification of Protein Expression: Total protein was extracted from treated cells using Cell Lysis Buffer from Promega Corp. 50 µg of proteins from each sample were separated by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto nitrocellulose membrane. The membrane was incubated overnight in cold condition with primary antibodies. The membrane was washed and incubated with horseradish peroxidase conjugated secondary antibody and the blots were developed using a chemiluminescent system.
The expression of total and phosphorylated (p-) mTOR targets, including ribosomal S6 (32 kDa), p-S6 (S235/236, 32 kDa) and mTORC2 direct and indirect targets, including Akt (60 kDa), p-Akt S473 (60 kDa), and GAPDH (house-keeping marker, 37 kDa) markers using antibodies were examined by Western blot analysis using proteins from treated cells. The apoptosis markers used include cleaved PARP, Bax, and Bcl2. Various pathways involved in cell apoptosis have been identified, encompassing mechanisms like the cytochrome c signaling and caspase family pathways (**Wang et al. 2017**). The Bcl-2 family of proteins plays a pivotal role in regulating apoptosis, a crucial process for maintaining tissue homeostasis and responding to various cellular stresses. Bcl-2 is a 26 kDa anti-apoptotic protein known for its ability to promote cell survival by

interacting with other members of the Bcl-2 family, such as Bax. to balance cell death and survival signals. Specifically, Bcl-2 functions by inhibiting the release of cytochrome-c from the mitochondria, thereby preventing the activation of the intrinsic apoptotic pathway (**Antonsson, 2004; Kluck et al. 1997**). On the other hand, Bax is a 21 kDa pro-apoptotic protein within the Bcl-2 family crucial to activate apoptosis via the intrinsic pathway (**Hockenberry et al. 1993**). Additionally, p53 plays a pivotal role in initiating apoptosis of cancer cells by activating multiple cell death pathways. Central to these processes is the activation of caspase-3, which cleaves various substrates including important cellular proteins like poly (ADP-ribose) polymerase (PARP) with molecular weight of 89 kDa, ultimately contributing to programmed cell death (**Wang et al. 2017**). Upon quantification of protein levels for each treatment group, western blots were performed to measure protein expression differences between each treatment group using the markers previously mentioned.

Electrocardiography: Electrocardiography (ECG) was employed in this study to assess cardiac function and detect DOX-induced cardiotoxicity in mice, as previously established in murine models using the Vevo 770TM imaging system (VisualSonics, Inc., Toronto, Canada) (**Das et al. 2010; Fisher et al. 2005**). A 30-MHz probe was used to obtain two-dimensional, M-mode, and Doppler imaging from parasternal short axis view at the level of papillary muscle and the apical four-chamber view. Left ventricular measurements were calculated using Vevo analysis software: LVIDd (Left ventricular end-diastolic diameter); LVIDs (Left ventricular end-systolic diameter); PWDT (Left ventricular end-diastolic posterior wall thickness); PWST (Left ventricular end-systolic posterior wall thickness); AWDT (Left ventricular end-diastolic anterior wall thickness); AWST (Left ventricular end-systolic anterior wall thickness); SV (Stroke volume); EF (Ejection fraction); FS (Fractional shortening); CO (Cardiac Output), HR (Heart Rate), E wave, and A wave (**Dodge. 2024; Gao et al. 2011**). Number of days survived and body weight were also recorded as baseline and weekly throughout the study. This approach ensured tracking of the DOX-induced cardiotoxicity comparing all treatment groups of the study.

1) **To determine the in vitro anticancer therapeutic potential of PDE5 and mTOR inhibition (Sild and Rapa respectively) in combination with DOX in TNBC cells.**

The additive effect of Sild and Rapa with DOX in an in vitro model was investigated. Full combination of DOX, Sild, and Rapa is anticipated to increase cancer cytotoxicity in TNBC cells more than DOX only treatment. The investigation explores PDE5 inhibition through Sild to increase DOX-induced cytotoxicity of breast cancer cells by increasing cGMP levels to stimulate PKG and decrease *beta*-catenin, which decreases cell proliferation. Likewise, mTOR inhibition through Rapa disrupts S6 phosphorylation to lessen cell proliferation. The drug combination is tested on triple negative human breast cancer cells (MDA-MB-231) and triple negative murine breast cancer cells (4T1) individually to ensure combinatorial effects with the DOX anticancer mechanism.

Experimental Protocol

The evaluation of the combined therapeutic efficacy involving DOX, Sild, and Rapa encompasses tests and assays detailed in **Figure 4 and 5** for MDA-MB-231 and 4T1, respectively.

Initially, the drug combination (DOX, Rapa, Sild) was tested on MDA-MB-231 and 4T1 cells to ensure cancer toxicity. Cells were cultured in RPMI medium (with 10% Fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS)). Cells were subjected to the following treatment groups:

(i) Control, (ii) DOX (1µM) alone, (iii) DOX+Rapa (100 nM)+Sild (10 µM), (iv) DOX+Sild, (v) DOX+Rapa, (vi) Rapa+Sild, (vii) Sild alone, (viii) Rapa alone. Cell death, Cell apoptosis, Cell viability, and Western Blot was performed to evaluate this objective.

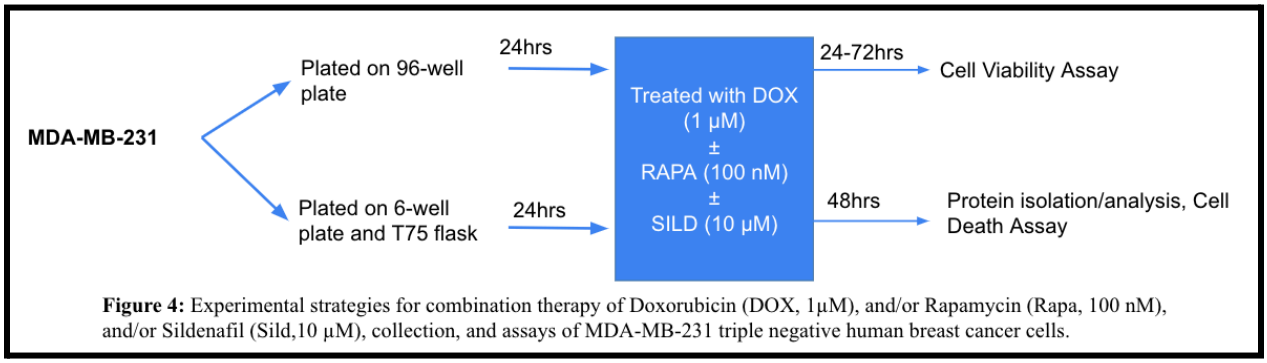

Figure 4: Experimental strategies for combination therapy of Doxorubicin (DOX, 1µM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 µM), collection, and assays of MDA-MB-231 triple negative human breast cancer cells.

As shown in **Figure 5**, for protein isolation and cell death study, MDA-MB-231 cells were treated for 48 hrs, but 4T1 cells were treated for 24 hrs, due to 4T1 rapid proliferation under normal conditions and cell death once treated.

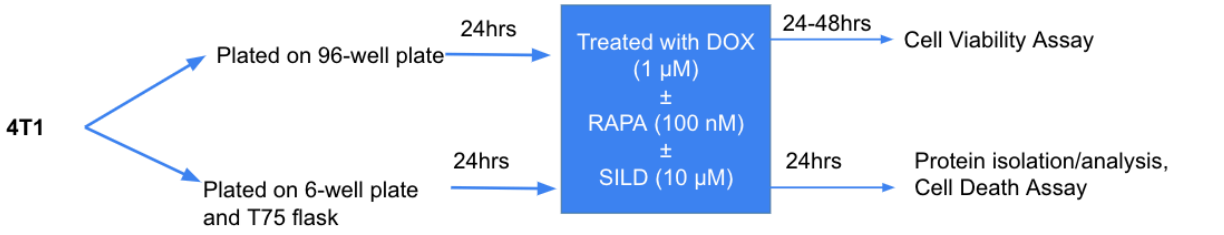

Figure 5: Experimental procedures for treating, collecting, and analyzing 4T1 triple negative murine breast cancer cells with combination therapy of Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 μM)

Results

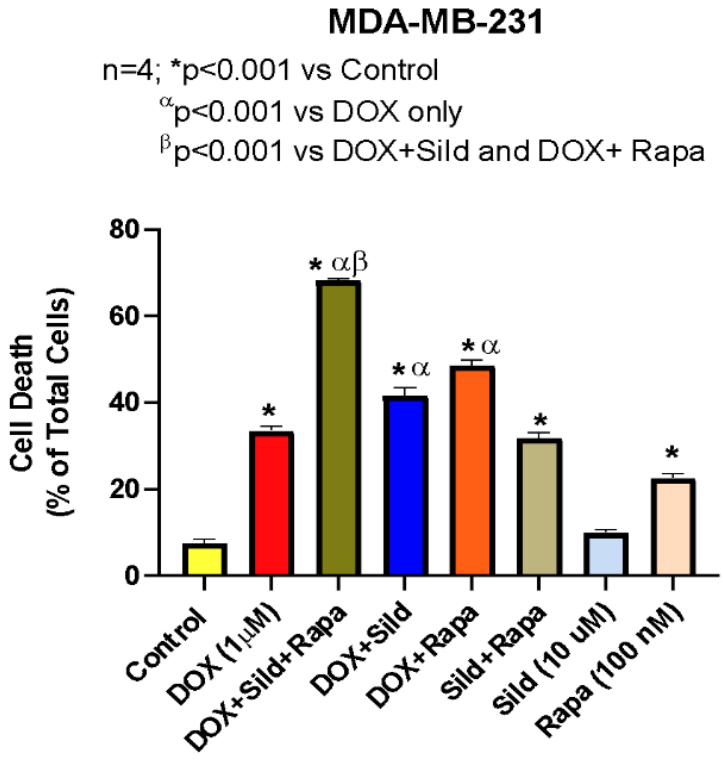

Figure 6: Effect of Combination Therapy on MDA-MB-231 Cell Death
Cell death of MDA-MB-231 human breast cancer cells treated for 48 hrs with Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 μM) using Trypan Blue exclusion assay, *p<0.001 vs. Control, αp< 0.001 vs. DOX only, βp<0.001 vs. DOX+Sild and DOX+Rapa; n=4

Cell death assessed by TB exclusion assay indicated DOX significantly killed MDA-MB-231 cells (**Figure 6**). DOX-induced cell death was further enhanced in the presence

of Sild or Rapa. Interestingly, combination of Sild and Rapa treatment in presence of DOX significantly stimulated DOX-induced cell death more than individual treatments. Treatment with Sild and Rapa in combination as well as Rapa alone also causes significant cell death in absence of DOX as compared to Control.

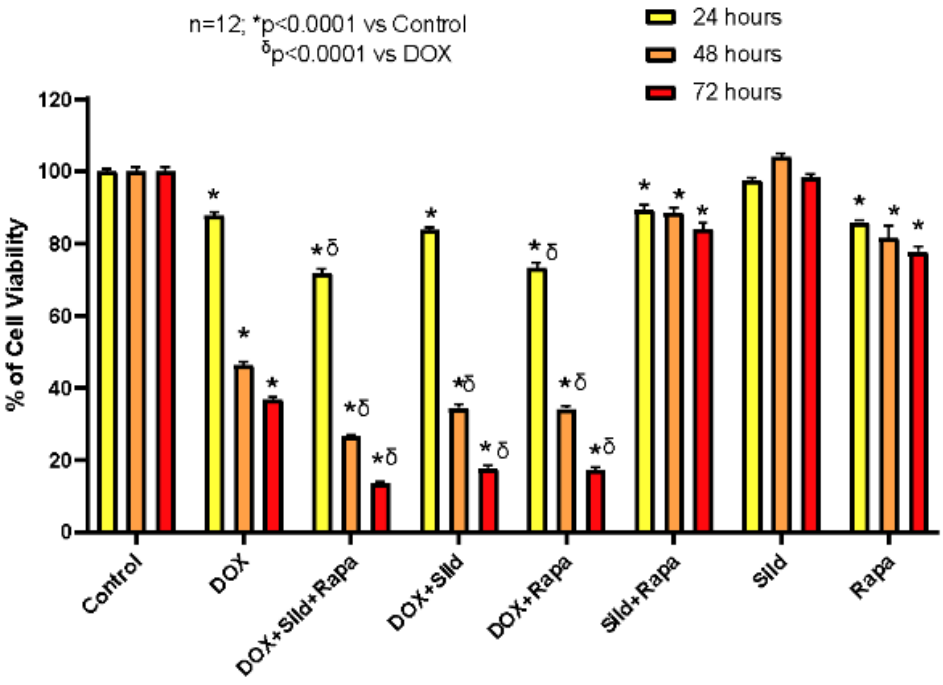

Figure 7: **Effect of Combination Therapy on MDA-MB-231 Cell Viability**
Cell viability of MDA-MB-231 human breast cancer cells treated with Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 μM) in 96-well plate for 24, 48, and 72 hrs using MTS assay; *p<0.0001 vs. Control, δp<0.0001 vs. DOX; n=12 per group.

Cell viability for MDA-MB-231 assessed by MTS assay was shown in **Figure 7** to indicate that after 24, 48, and 72 hrs of treatment, all groups except Sild alone treatment resulted in significantly less cell viability than that of control group. The full combination (DOX+Sild+Rapa) along with DOX+Sild and DOX+Rapa had significantly reduced cell viability compared to DOX alone treatment (**Figure 7**).

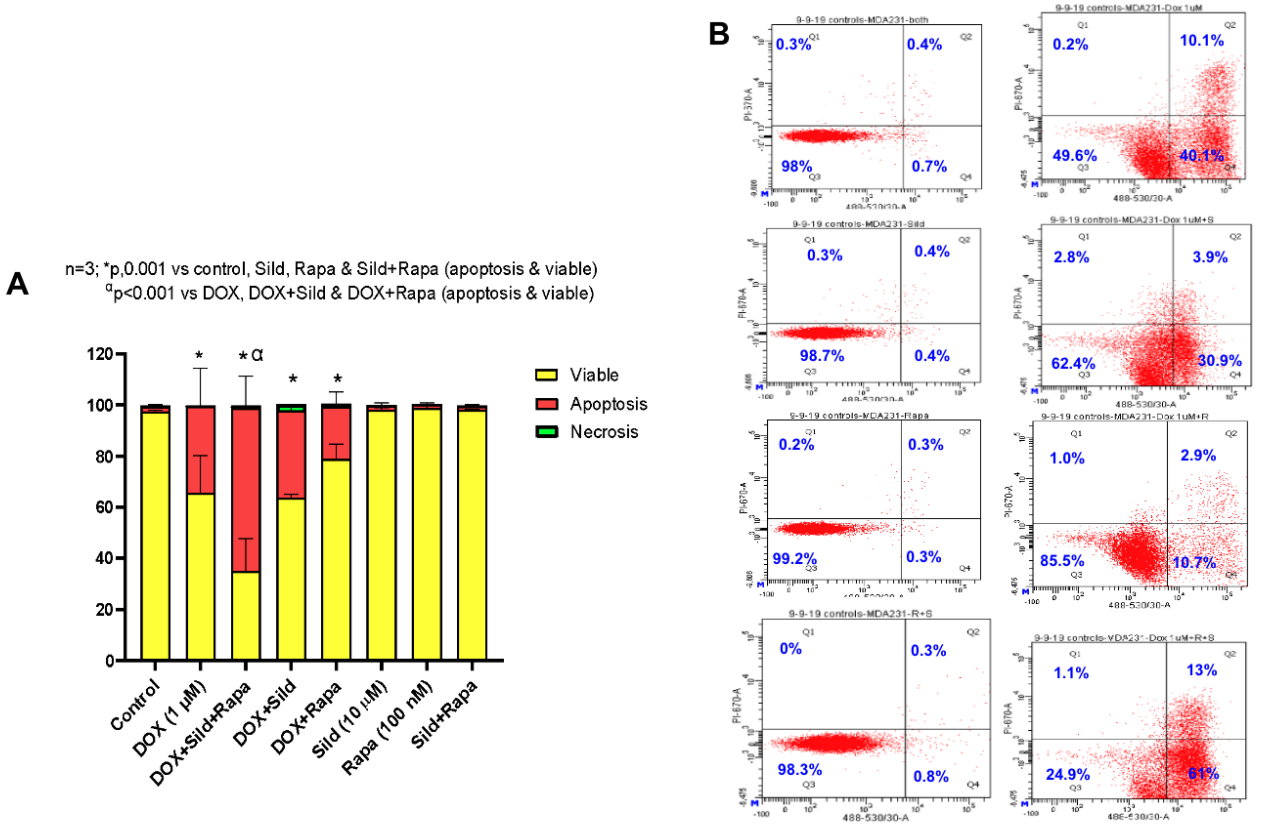

Figure 8: Effect of Combination Therapy on MDA-MB-231 Cell Apoptosis and Necrosis
Sild and Rapa potentiate doxorubicin (DOX)-induced cell death of MDA-MB-231 human breast cancer cells after 48 hrs of treatment. (A) Quantification of viable, apoptosis and necrosis and (B) Representative flow cytometry charts illustrating Q1 should be necrosis, Q2+Q4: apoptosis and Q3: viable

Quantitative analysis depicted in **Figure 8A** delineates the distribution of viable, apoptotic, and necrotic cells across the experimental groups after 48 hrs of treatment, showcasing the heightened apoptotic activity associated with DOX alone and even greater apoptosis with the DOX+Sild+Rapa combination, while negligible necrosis is observed across all treatment groups. The representative images of flow cytometry data with DOX only treatment reveal a large percentage of apoptotic cells in quadrant 2 and 4 (Q2 and Q4) and less viable cells in quadrant 3 (Q3) (**Figure 8**). The full combination indicates an even greater percentage of cells in both early (Q4) and late apoptosis (Q2) and less viable cells (Q3) compared to DOX alone treatment (**Figure 8B**).

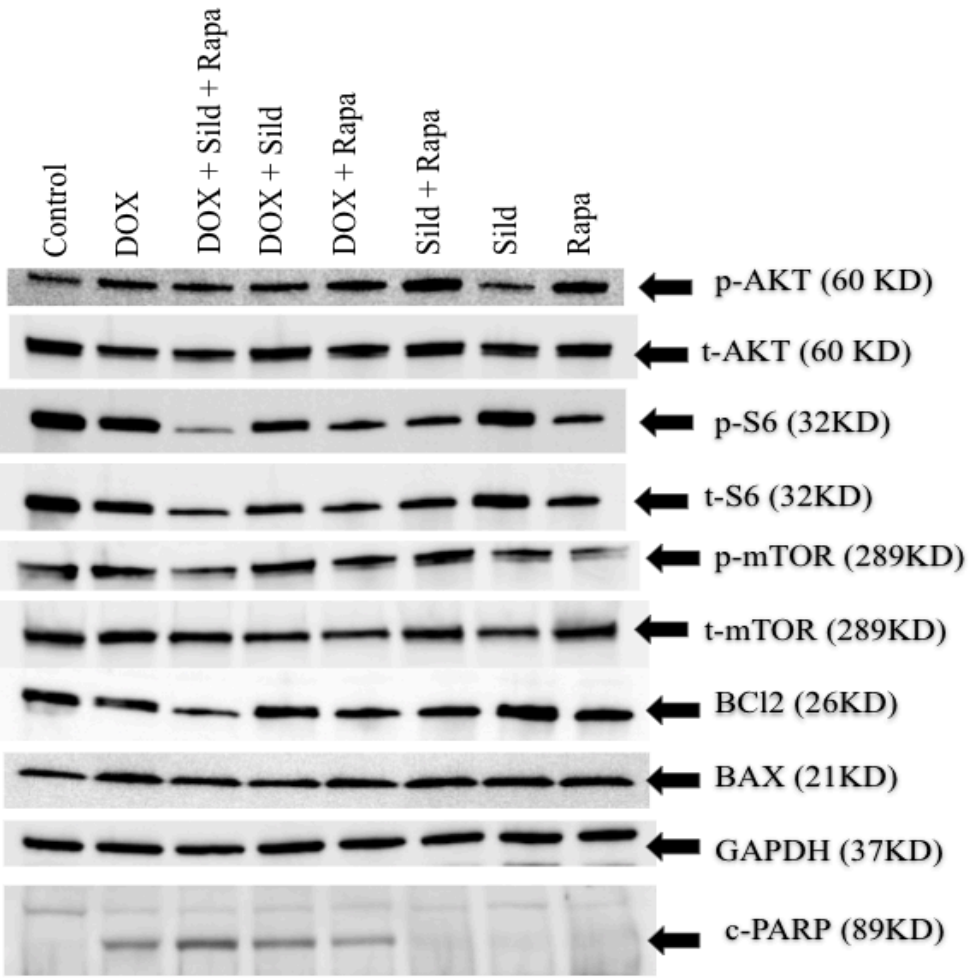

Figure 9: **Effect of Combination Therapy on MDA-MB-231 Protein Expression** Chemiluminescence Images of Western Blots for MDA-MB-231 human breast cancer cells treated with Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 μM) in T75 flasks for 48 hrs.

Moreover, protein expression as illustrated in **Figure 9**, confirms these results; DOX+Sild+Rapa treatment is associated with decreased phosphorylation levels of S6 and mTOR, and expression of Bcl2 for MDA-MB-231 compared to DOX only. Conversely, the combination shows elevated expression of cleaved PARP as compared to DOX alone (**Figure 9**). The mTOR and S6 exhibit less phosphorylation with combination treatment (DOX+Sild+Rapa) indicated inhibition of mTOR activity, specifically mTORC1, which could attenuate cancer cell proliferation. DOX treatment reduced expression of Bcl-2 (anti-apoptotic protein) and increased the cleaved PARP as compared to control and Sild/Rapa, which was further elevated with

combination treatment with DOX+Sild+Rapa as compared to DOX alone in TNBC cells (**Figure 9**).

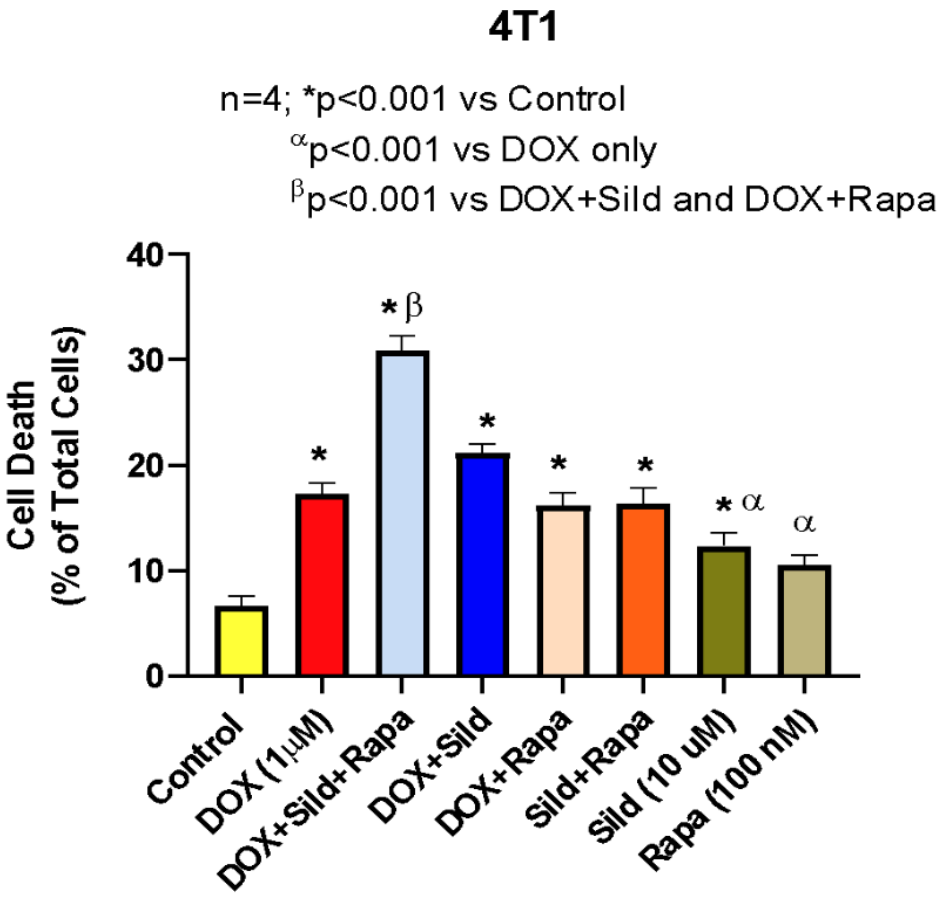

Figure 10: Effect of Combination Therapy on 4T1 Cell Death
Cell death of 4T1 murine breast cancer cells treated for 24 hrs with Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 μM) using Trypan Blue exclusion assay, *p<0.001 vs. Control, αp< 0.001 vs. DOX only, βp<0.001 vs. DOX+Sild and DOX+Rapa; n=4

Murine TNBC cells were also treated for 48 hrs and analyzed via TB exclusion assay. All treatment groups, with the exception of Rapa alone, show increased cell death after 24 hrs relative to the control group (**Figure 10**). Furthermore, **Figure 10** illustrates that when Sild and Rapa are administered individually, they result in notably higher cell death compared to DOX alone in 4T1 murine breast cancer cells. The combination of Sild and Rapa significantly boosts DOX-induced cell death, surpassing the effects seen with DOX combined with either Sild or Rapa alone (**Figure 10**).

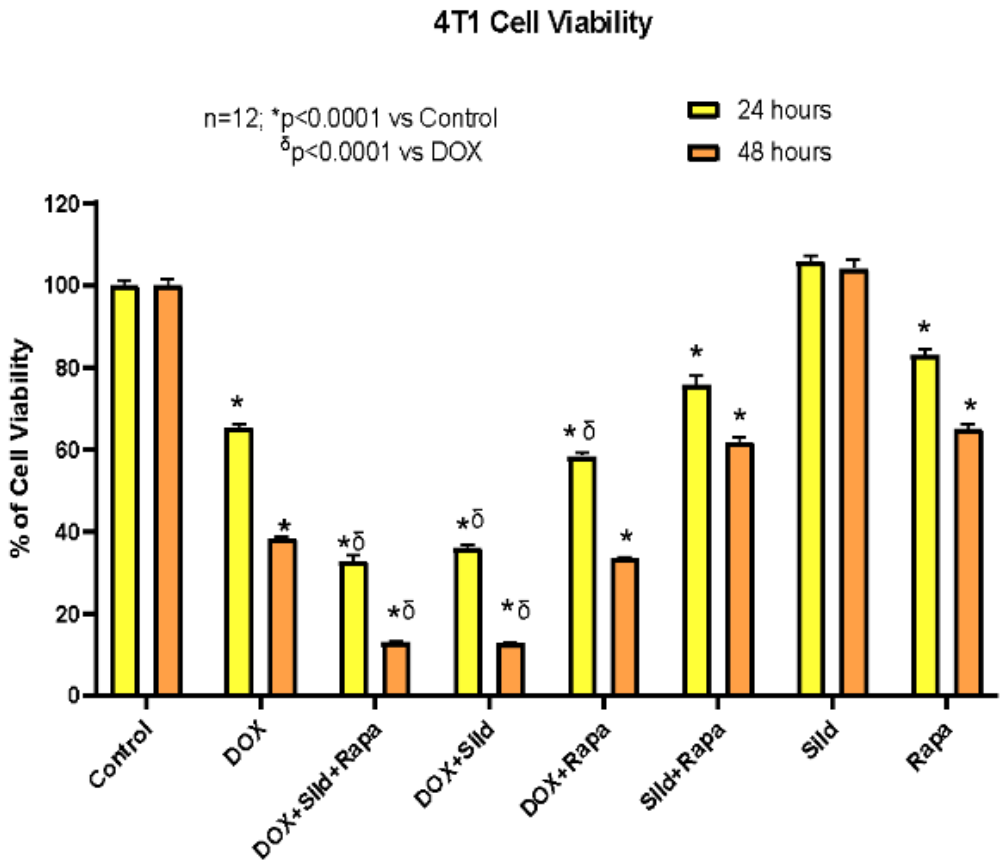

Figure 11: **Effect of Combination Therapy on 4T1 Cell Viability**
Cell viability of 4T1 breast cancer cells treated with Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 μM) in 96-well plate for 24 and 48 hrs using MTS assay; *p<0.0001 vs. Control, ᵟp<0.0001 vs. DOX; n=12 per group.

The MTS assay of 4T1 cells depicted in **Figure 11**, was used to evaluate cell viability. Results showed that, with the exception of Sild alone, all treatment groups exhibited significantly lower cell viability compared to the control group. The combination treatments, as well as DOX+Sild and DOX+Rapa, led to reduced cell viability compared to DOX alone between 24 and 48 hrs (**Figure 11**).

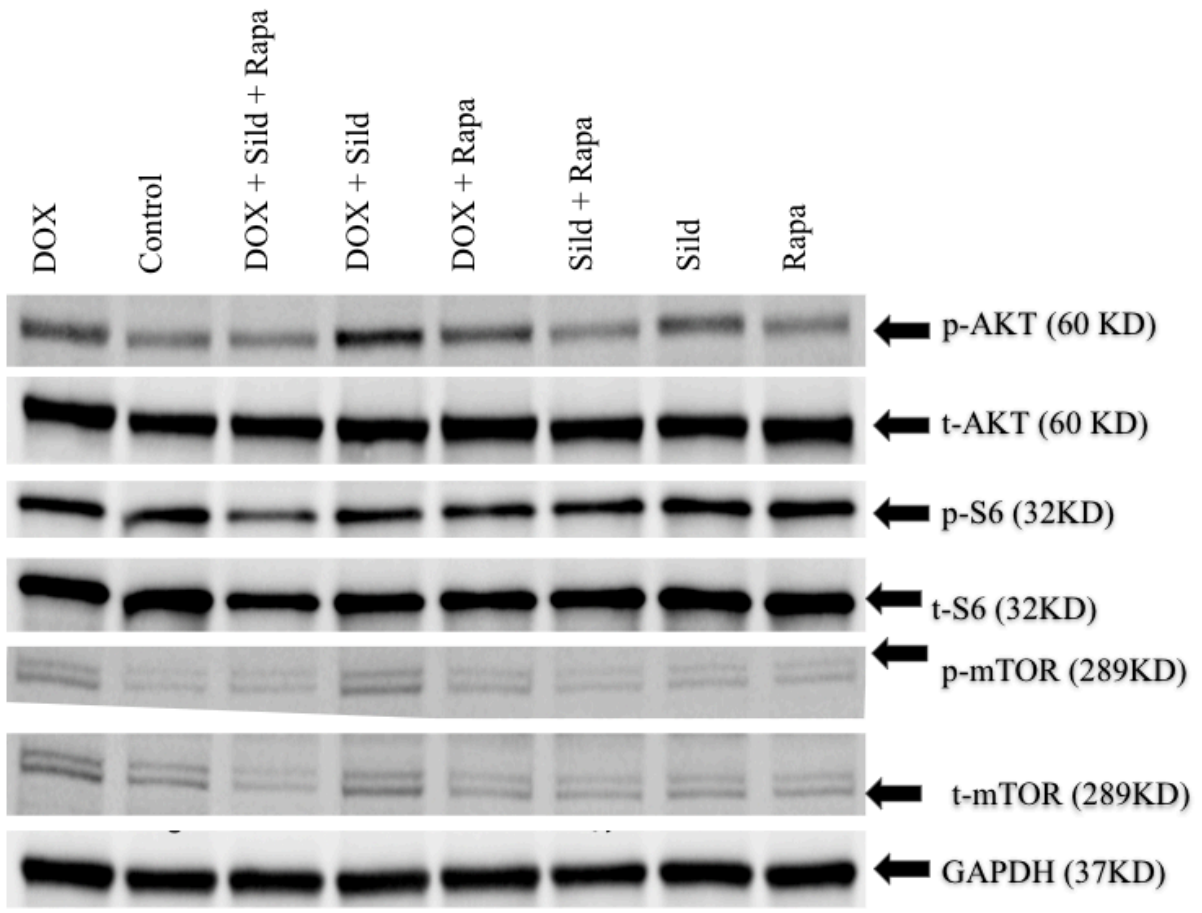

Figure 12: Effect of Combination Therapy on 4T1 Protein Expression
Chemiluminescence Images of Western Blots for 4T1 murine breast cancer cells treated with Doxorubicin (DOX, 1μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 μM) in T75 flasks for 48 hrs.

After 24 hrs of treatment with DOX, 4T1 murine breast cancer cells revealed increased expression of p-AKT, and p-mTOR as shown in **Figure 12**. As illustrated in **Figure 12**, DOX+Sild+Rapa, 4T1 murine breast cancer cells exhibited reduced phosphorylation levels of S6 and mTOR, compared to cells treated with DOX alone. This study demonstrates in both triple negative breast cancer cells, 4T1 (murine) and MDA-MB-231(human), Sild and Rapa augment DOX-induced cell death and apoptosis. This underscores the superiority of the DOX+Sild+Rapa combination in promoting cell death and apoptosis for TNBC models.

2) **To examine the effects of PDE5 and mTOR inhibition (Sild and Rapa respectively) on anticancer efficacy of DOX and ErbB2 inhibitor (AG825) in ErbB2-positive breast cancer cells, while protecting cardiomyocytes (AC-16).**

The ERBB family includes four tyrosine kinase receptors (ErbB1-4) that dimerize upon ligand binding, activating PI3K/AKT, Ras/Raf/MAPK, and JAK/STAT pathways (**Zhao et al. 1998**). ErbB2 has an intact kinase domain but cannot bind ligands, resembling ligand-bound receptors. Despite this, ErbB2 amplifies signaling by dimerizing with other ErbB receptors to amplify extracellular input (**Sysa-Shah et al. 2012**). Additionally, human ErbB2-positive breast cancer is characterized by high metastasis and drug resistance. There seems to be clear benefits from DOX in patients with ErbB2 positive breast cancer. However, when optimizing DOX-induced cytotoxicity, ErbB2 inhibitors induce additive apoptotic signaling, which leads to increased ROS generation and cardiac dysfunction (**Pentassuglia et al. 2007**). We examined whether Sild and Rapa enhance the cytotoxicities of a DOX and ErbB2 inhibitor, while protecting cardiomyocytes.

To test this goal, the drug combination in combination with DOX and AG825 (chemical inhibitor of ErbB2) was treated on spheroid culture of double-negative, ErbB2 positive human breast cancer cells (SKBr3) compared to the DOX and AG825 group. This ensures effective ErbB2 targeted therapy to kill the SKBr3 cells. To examine how the combination treatment successfully kills the cancer cells but prevents chemo-immunotherapy- induced cardiotoxicity, human cardiomyocytes (AC16) were exposed to the culture medium derived from SKBr3 cells, treated with the drug combination ± ErbB2 inhibitor. This experiment offers a comprehensive understanding of the mechanisms associated with novel factors released from DOX and AG825-treated cancer cells, which mediate cardiotoxicity. As previously mentioned, the combination therapy works to decrease DOX-induced cytotoxicity of AC16 via opening mitochondrial K_{ATP} channels. In cardiomyocytes, mTOR inhibition works through the AKT/S6 pathways to increase cell survival. Previous research has studied how Sild and Rapa impact cancer and cardiomyocyte apoptosis independently. However, this is a novel approach to examine whether co-treatment of Sild and Rapa could enhance cytotoxic effect of DOX on cancer cells through distinct yet complementary mechanisms, while exhibiting protective properties towards cardiomyocytes, possibly due to differences in metabolic activity and

signaling pathways between cancer cells and normal cardiomyocytes. This selectivity may arise from variations in drug uptake, metabolism, or expression of drug targets, thereby safeguarding cardiomyocytes from the harmful effects of these agents.

a) **The cytotoxicity of a DOX and ErbB2 inhibitor with co-treatment of Sild and Rapa will be examined on double negative, ErbB2+ human breast cancer cells.**

Experimental Protocol

To examine this relationship, SKBr3 cells (ErbB2 positive, Estrogen and Progesterone receptors negative breast cancer cells) were cultured in RPMI medium (with 10% FBS and 1% PS). When experimenting with SKBr3, AG825, a chemical ErbB2 inhibitor, was simultaneously utilized with DOX (**Fiszman & Jasnis, 2011**). The evaluation of the combined therapeutic efficacy involving DOX, AG-825, Sild, and Rapa encompassed tests and assays detailed in **Figure 13** for SKBr3 cells treated in the following treatment groups:
(i) Control, (ii) DOX(1µM) alone, (iii) AG825(5µM) alone, (iv) DOX+ AG825, (v) DOX+AG-825+Rapa (100 nM) +Sild (10 µM), (vi) DOX+AG825+Sild, (vii) DOX+AG825+Rapa, (viii) Rapa+Sild. Cell death, cell viability, and western blots were performed to quantify and visualize the differences between each treatment group.

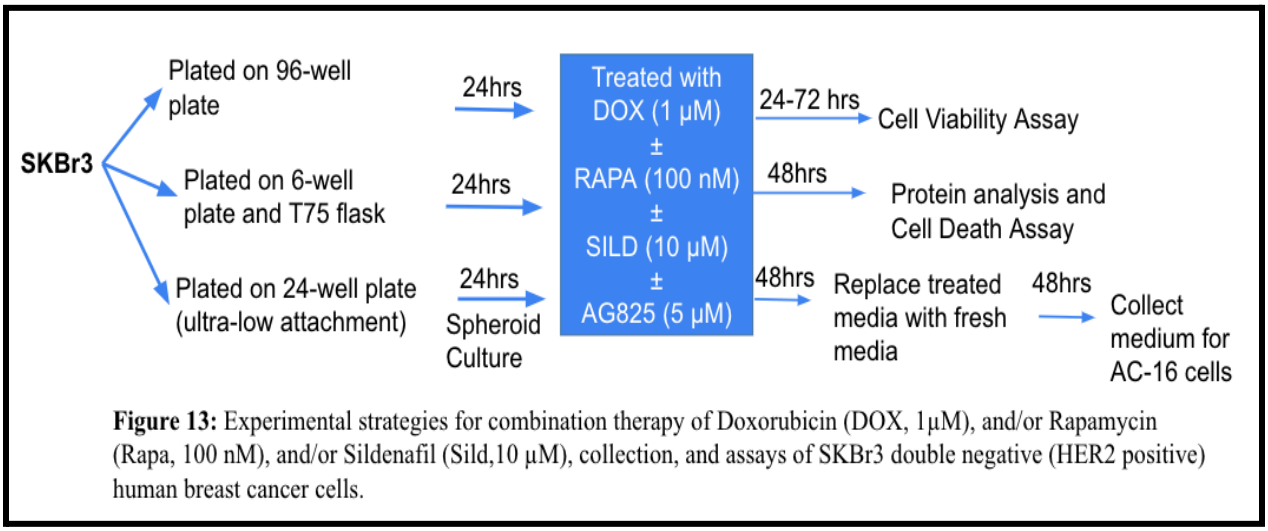

Figure 13: Experimental strategies for combination therapy of Doxorubicin (DOX, 1µM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 µM), collection, and assays of SKBr3 double negative (HER2 positive) human breast cancer cells.

Results

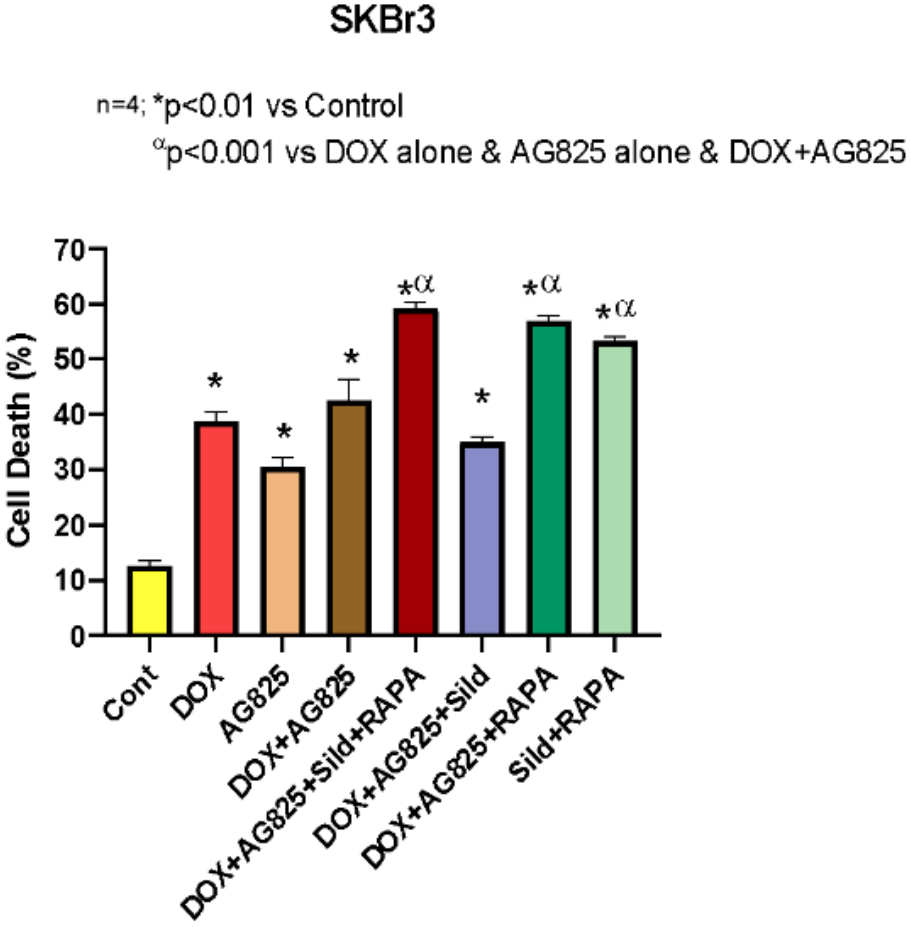

Figure 14: Effect of Combination Therapy on SKBr3 Cell Death
Cell death of SKBr3 human breast cancer cells treated for 48 hrs with Doxorubicin (DOX, 1μM), and/or AG825 (5μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 μM) using Trypan Blue exclusion assay, *p<0.01 vs. Control, αp< 0.001 vs. DOX alone and AG825 alone and DOX+AG825; n=4

Expanding our investigation to ErbB2-positive breast cancer cells (SKBr3), we found that the combined treatment of DOX, AG825, Sild, and Rapa similarly enhanced cell death after 48 hrs (**Figure 15**, lead to less viable cells over 24 to 72 hrs of treatment and altered protein expression (**Figure 16**). The combined regimen exhibits heightened cell death when subjected to the combined treatment of DOX, AG825, Sild, and Rapa after 48 hrs, demonstrating statistically significant outcomes (**Figure 14**). Full combination- DOX+ AG825+ Sild+ Rapa,

DOX+AG825+Rapa and Sild+Rapa had notably greater cell death than DOX alone, AG825 alone, and DOX+AG825 (**Figure 14**).

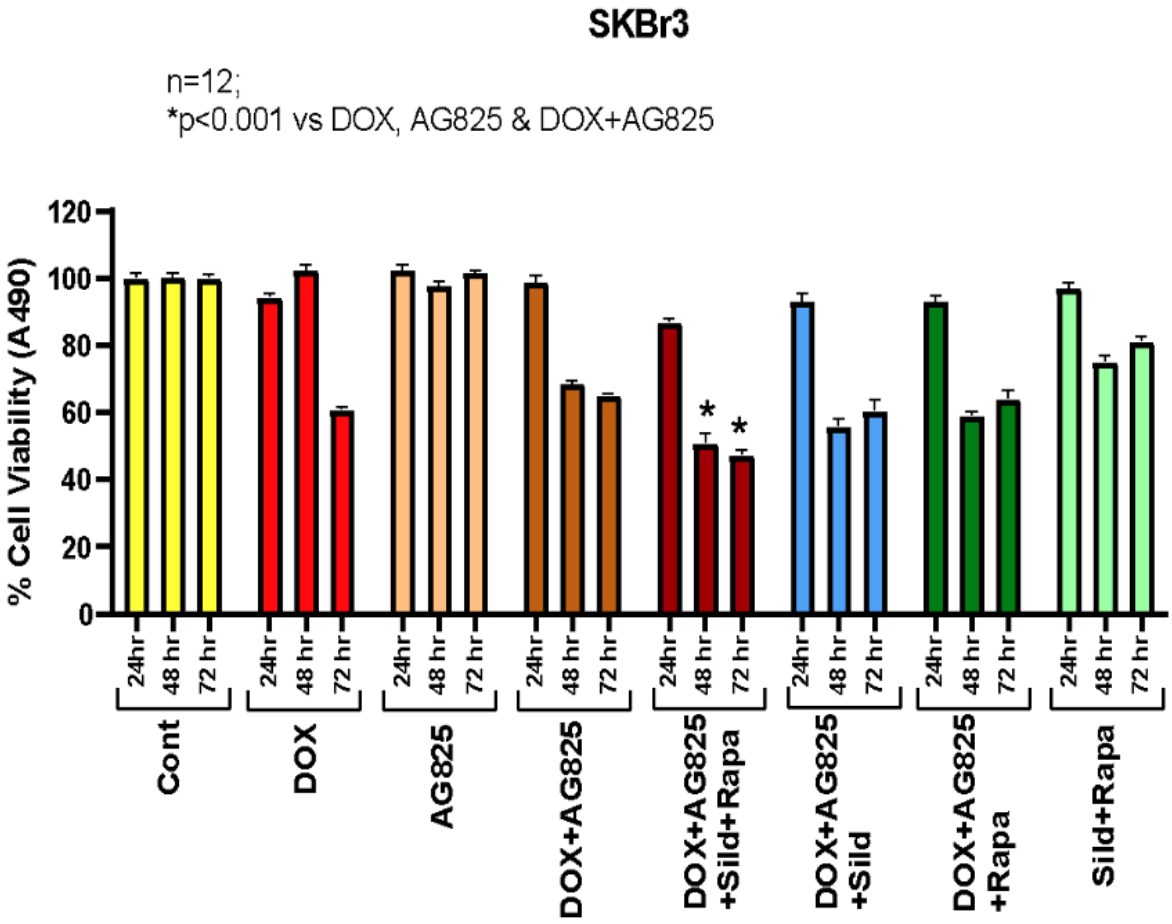

Figure 15: Effect of Combination Therapy on SKBr3 Cell Viability
Cell viability of SKBr3 breast cancer cells treated with Doxorubicin (DOX, 1µM), and/or AG825 (5µM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 µM) in 96-well plate for 24, 48, and 72 hrs using MTS assay; *p<0.001 vs. DOX, AG825, and DOX+AG825; n=12 per group.

In accordance with observations of MDA-MB-231 cells, double negative breast cancer cells (SKBr3) treated with the comprehensive combination therapy induce a significantly greater reduction in cell survival over the period of 24 to 72 hrs was assessed by MTS assay, compared to DOX alone and DOX+AG825 as displayed in **Figure 15**.

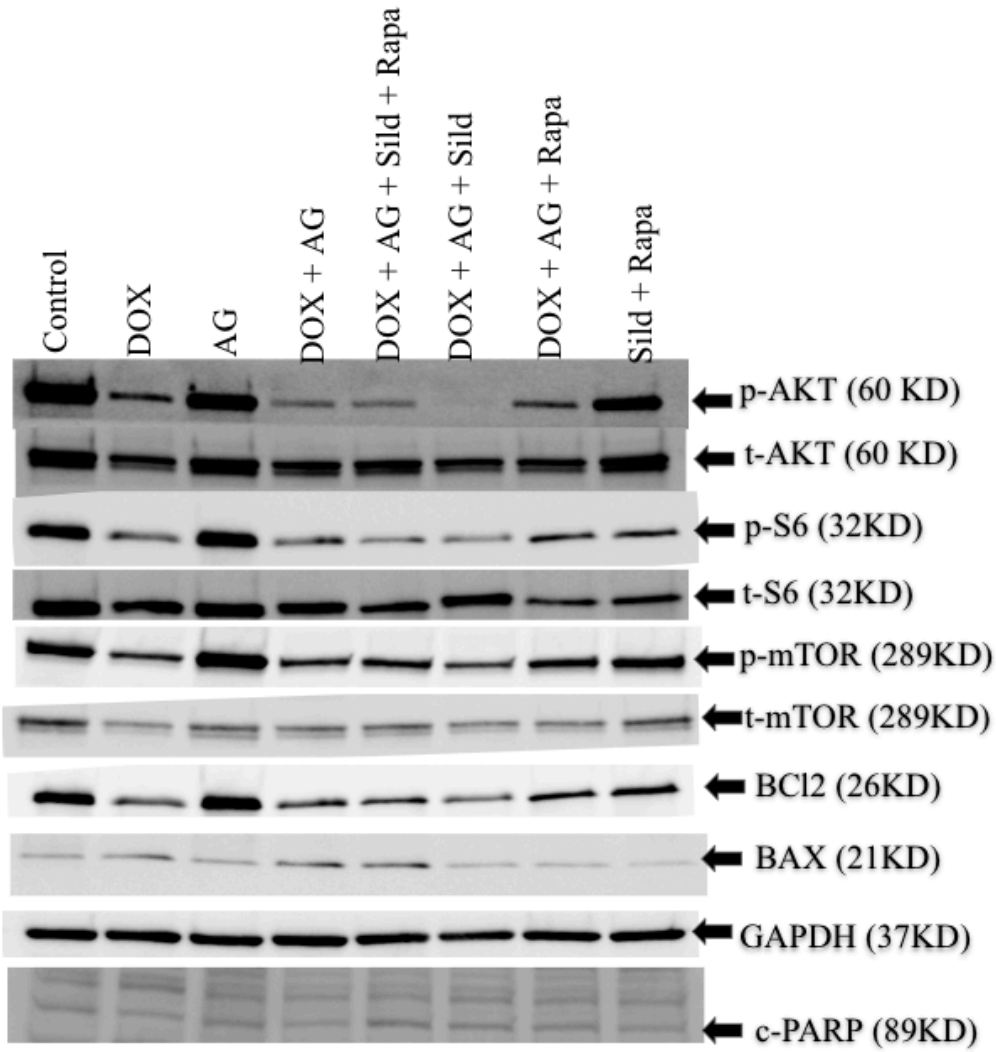

Figure 16: **Effect of Combination Therapy on SKBr3 Protein Expression**
Chemiluminescence Images of Western Blots for SKBr3 human breast cancer cells treated with Doxorubicin (DOX, 1μM), and/or AG825 (5μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 μM) in T75 flasks for 48 hrs.

Furthermore, analysis of protein expression in **Figure 16** reveals consistent findings of SKBr3 cells with those observed in MDA-MB-231 cells, wherein the DOX+AG825+Sild+Rapa combination leads to decreased levels of p-S6 compared to DOX+AG825. Additionally, levels of p-mTOR and the anti-apoptotic protein Bcl2 were lower for the full combination compared to treatment with DOX and AG825. DOX+AG825+Sild+Rapa combination increased the level of BAX and cleaved PARP in SKBr3 cells compared to the DOX and AG825 group. Based on the

data, Sild and Rapa enhanced the cytotoxicity of chemo- and immunotherapy in ErbB2 positive breast cancer.

b) To examine the cardioprotective effect of Sild and Rapa against chemo- and immunotherapy-induced cytotoxicities in human cardiomyocytes.

AC-16 cells, originating from the fusion of primary cells sourced from adult human ventricular heart tissues, were used to test the combination therapy effect on the heart (**Jiang et al. 2018**). Sild and Rapa were evaluated for the release of cardiotoxic factors from breast cancer spheroids after treatment with DOX and ErbB2 inhibitors.

Experimental Protocol

SKBr3 cells formed spheroids by plating in ultra-low attachment surface polystyrene 24-well plates for 72 hrs. Then cells were treated for 48 hrs with the drugs (as proposed in Part 2A) and then treated medium was replaced with fresh medium for 48 hrs incubation. Then medium was collected to treat human cardiomyocytes as shown in **Figure 17**. AC-16 cells were cultured in DMEM medium (with 10% FBS+1% PS) and treated with the medium (20%) from culture medium derived from SKBr3 cells, treated with the drug combination ± ErbB2 inhibitor, in the following treatment groups:

(i) Control, (ii) DOX(1µM) alone, (iii) AG825(5µM) alone, (iv) DOX+ AG825, (v) DOX+AG825+Rapa (100 nM) +Sild (10 µM), (vi) DOX+AG82+Sild, (vii) DOX+AG82+Rapa, (viii) Rapa+Sild. Cell death, and western blots were performed to quantify the differences between each treatment group.

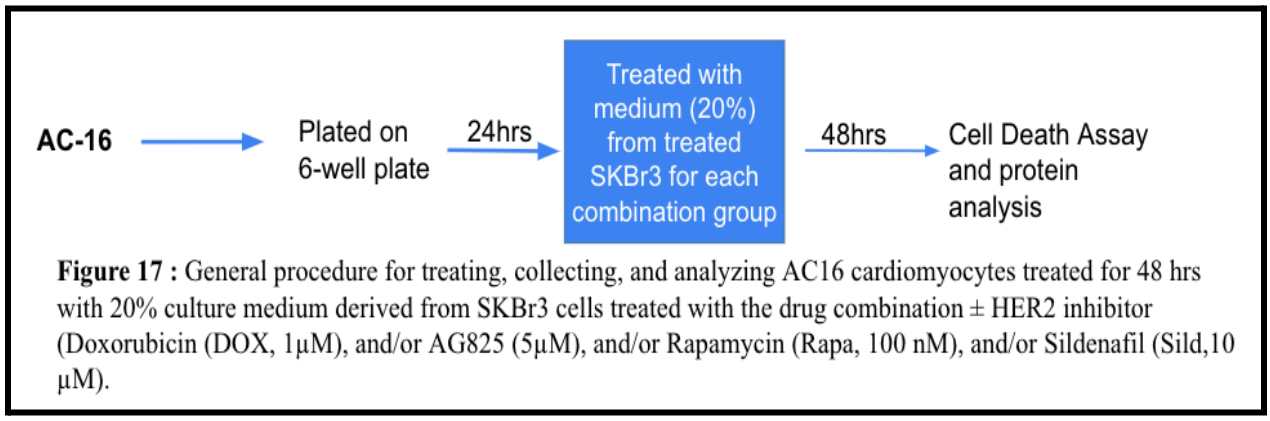

Figure 17 : General procedure for treating, collecting, and analyzing AC16 cardiomyocytes treated for 48 hrs with 20% culture medium derived from SKBr3 cells treated with the drug combination ± HER2 inhibitor (Doxorubicin (DOX, 1µM), and/or AG825 (5µM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 µM).

Results

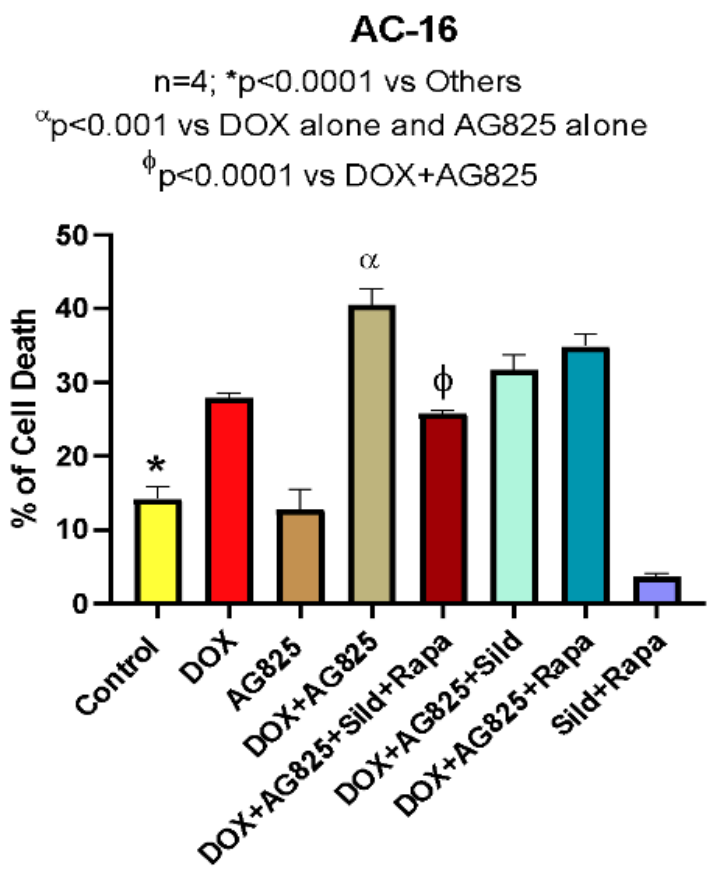

Figure 18: **Effect of Combination Therapy on AC16 Cell Death**
Cell death of AC16 human cardiomyocytes treated for 48 hrs with 20% culture medium derived from SKBr3 cells treated with the drug combination ± HER2 inhibitor (Doxorubicin (DOX, 1μM), and/or AG825 (5μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild,10 μM) using Trypan Blue exclusion assay, *p<0.0001 vs. Others, αp< 0.001 vs. DOX alone and AG825 alone, ϕp<0.0001 vs. DOX+AG825; n=4

Additionally, our study explores the protective effects of Sild and Rapa on cardiomyocytes exposed to chemotherapy and immunotherapy. In contrast to results of breast cancer models, analysis depicted in **Figure 18** reveals that human cardiomyocytes treated with medium from cancer cell spheroids treated with DOX+AG825 exhibit more cell death compared to treatment with DOX and AG825 independently. Additionally, Sild and Rapa co-treatment of cardiomyocytes with DOX for 48 hrs reveals significantly less cell death than that of DOX+AG825 group. Our data suggest that this combination regimen may mitigate cytotoxicity

against cardiomyocytes induced by factors released from cancer cells during treatment (**Figure 18**).

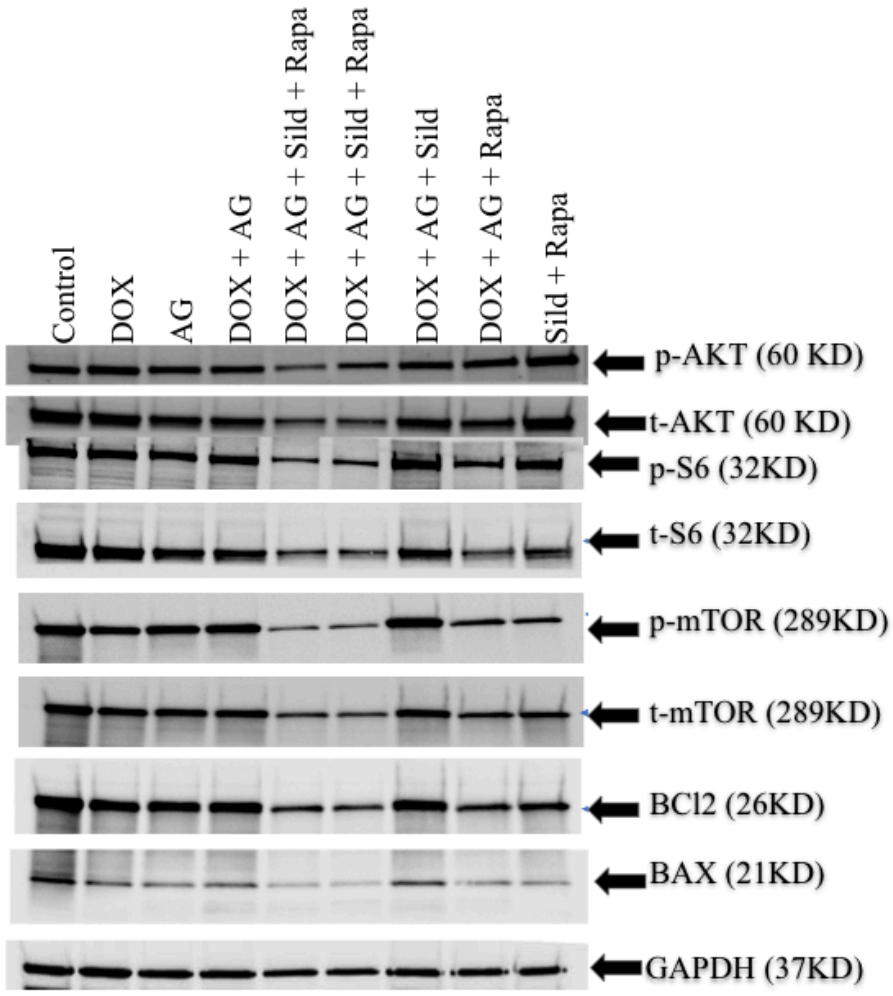

Figure 19: **Effect of Combination Therapy on AC16 Protein Expression**
Chemiluminescence Images of Western Blots for AC16 human cardiomyocytes treated with 20% culture medium derived from SKBr3 cells, treated with the drug combination ± HER2 inhibitor (Doxorubicin (DOX, 1μM), and/or AG825 (5μM), and/or Rapamycin (Rapa, 100 nM), and/or Sildenafil (Sild, 10 μM) in T75 flasks for 48 hrs.

Protein expression analysis in **Figure 19** reveals that the medium of SKBr3 combined treatment involving full combination regimen (DOX+AG825+Sild+Rapa) mitigates the phosphorylation levels of p-S6 and p-mTOR of AC16 cells in contrast to the administration of DOX and AG825. Furthermore, this combination regimen correlates with a reduction in the expression levels of the apoptotic protein BAX relative to treatment with DOX and AG825 group. Based on the results, Sild and Rapa protect cardiomyocytes against chemo- and

immunotherapy–induced cell death potentially by regulating different cytotoxic factors released from cancer cells.

3) To utilize a murine model to test the cardiac impact of combination therapy of DOX and Afatinib (ErbB2 inhibitor) with/without Sild and Rapa.

Trastuzumab was not tested in this study due to its specific targeting of ErbB2 and reliance on ligand binding for efficacy, which ErbB2 lacks. Instead, Afatinib was selected for mice testing because it inhibits multiple ErbB receptors, including ErbB2, regardless of ligand binding status. This broader spectrum of action is beneficial for studying the effects of ErbB inhibition in diverse contexts, such as cancer and cardiac development, where ErbB2 signaling plays critical roles beyond ligand binding alone (**Lewis et al. 2022)**. After determining the impact of combination therapy on an in vitro model, Sild and Rapa in combination with DOX was tested to observe if the combination could protect cardiac function following the chemo- and immuno- therapy on female C57/B6 mice for 12 weeks better than DOX and Afatinib group. This experiment showcases the outcomes of the drug combination in an *in vivo* model by body weight, survival rate, and echocardiography analysis.

Experimental Protocol

As shown in **Figure 20**, to examine the cardioprotective effect of combination treatment, female wildtype C57/BL6 mice were treated with Rapa (0.25 mg/kg/twice a week, i.p.) and Sild (0.4 mg/ml in drinking water) for 12 weeks. After 1 week of starting Rapa and Sild treatment, the mice were treated with DOX (6 mg/kg/ twice a week, for 2 weeks, i.p.). After a 1-week interval, mice were treated with Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks). After 4 weeks of completion of DOX and Afatinib treatment, cardiac function was monitored. After sacrificing the mice, blood and heart samples were collected for further molecular studies. Additional statistics include body weight and days of survival for each subject. For an *in vivo* perspective, cardiac echo analysis was carried out on treated mice, providing a non-invasive means to assess cardiac function and differences amongst treatment groups. The mice were treated as the following eight groups: (i) Control, (ii) DOXalone, (iii) AFA alone, (iv) DOX+

AFA, (v) DOX+AFA+Rapa+Sild, (vi) DOX+AFA+Sild, (vii) DOX+AFA+Rapa, (viii) Rapa+Sild.

The animal study was performed in accordance with the guidelines presented by Virginia Commonwealth University and the National Institute of Health (NIH) "Guide for the Care and Use of Laboratory Animals".

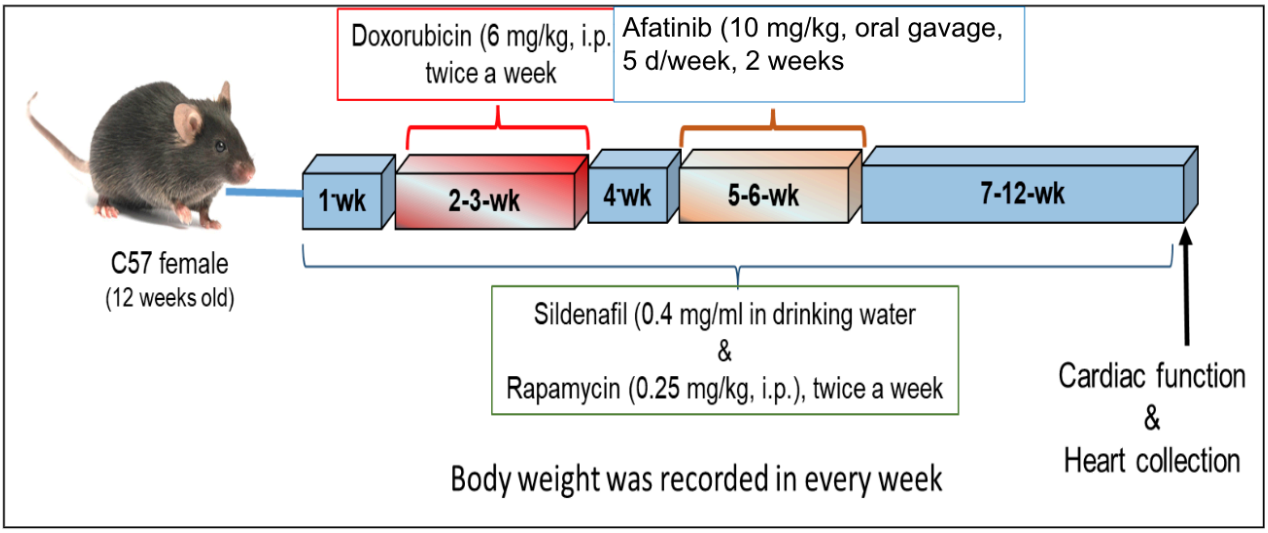

Figure 20: **Experiment Protocol for In Vivo Study with Combination Therapy**
General Procedure for treating female wild type C57/BL6 mice with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/twice a week, for 2 weeks, i.p.) and/or Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks) for 12 weeks while monitoring body weight and cardiac function.

Results

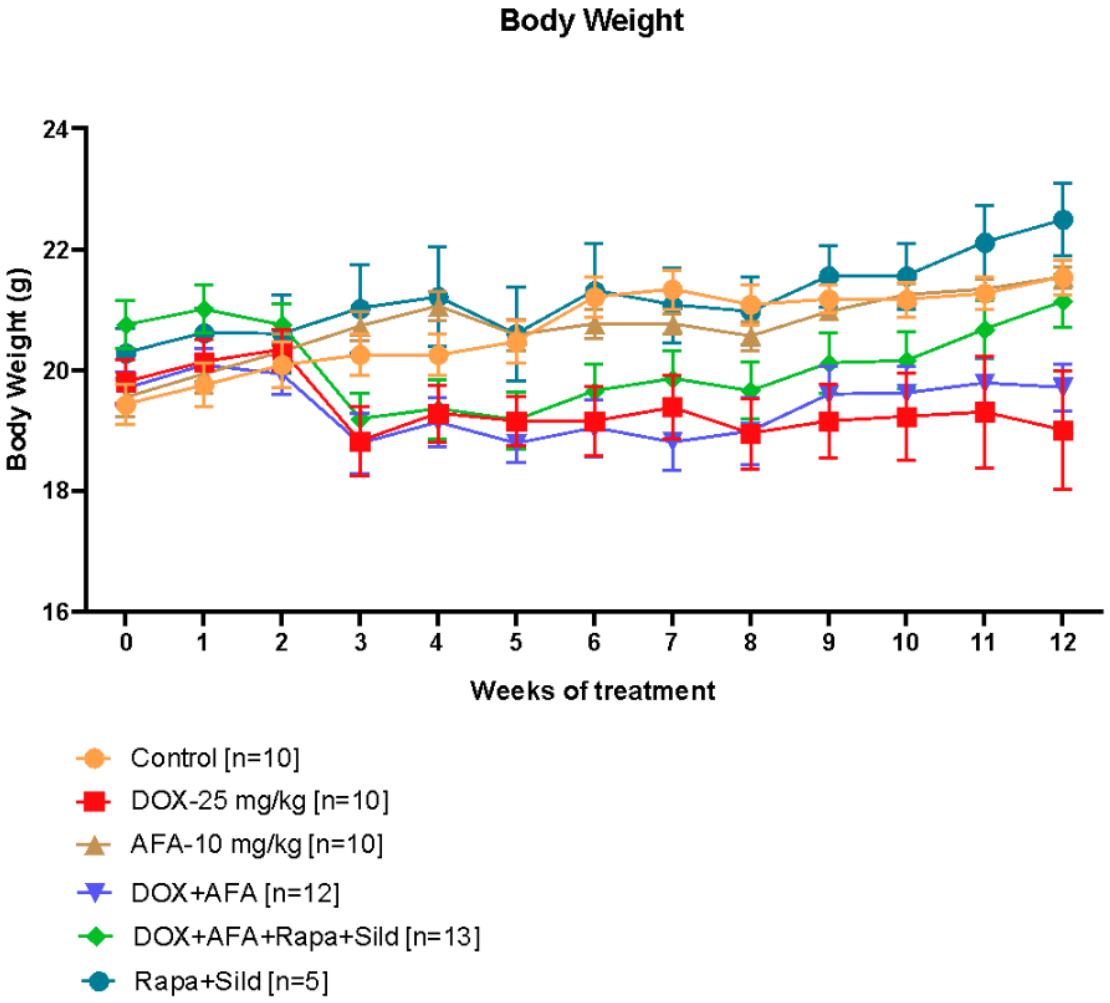

Figure 21: Effect of Combination Therapy on Body Weight
Body weight (weekly) of female C57/BL6 mice during the treatment with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/ twice a week, for 2 weeks, i.p.) and/or Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks).

Following the administration of DOX+AFA, a significant reduction in body weight was observed (**Figure 21**) among the mice, adversely impacting their survival rates shown in **Figure 22**. Mice subjected to combination treatment of DOX, AFA, Rapa, and Sild exhibited higher body weights compared to those treated solely with DOX and/or AFA; these mice displayed a less pronounced initial decline in body weight, which subsequently stabilized.

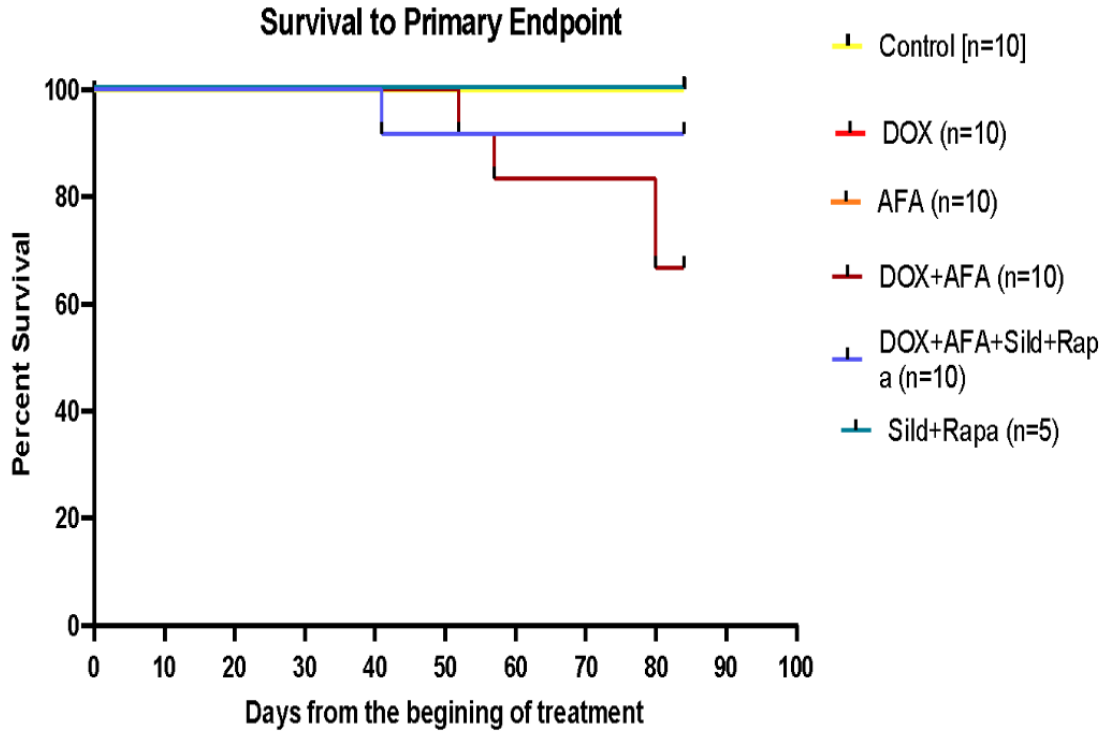

Figure 22: **Effect of Combination Therapy on Percent Survival**
Survival of female C57/BL6 mice days after beginning of treatment with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/ twice a week, for 2 weeks, i.p.) and/or Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks) throughout 12 week study.

The survival of mice treated with DOX and DOX+AFA declined in the second half of treatment compared to the control group as displayed in **Figure 22**. The full combination of DOX+AFA+Sild+Rapa resulted in an initial decrease in percent survival that stabilized halfway during the study period. **Figure 21 and 22** confirms that the cohort receiving the full combination regimen demonstrated less weight loss and prolonged survival respectively compared to those treated exclusively with DOX+ AFA.

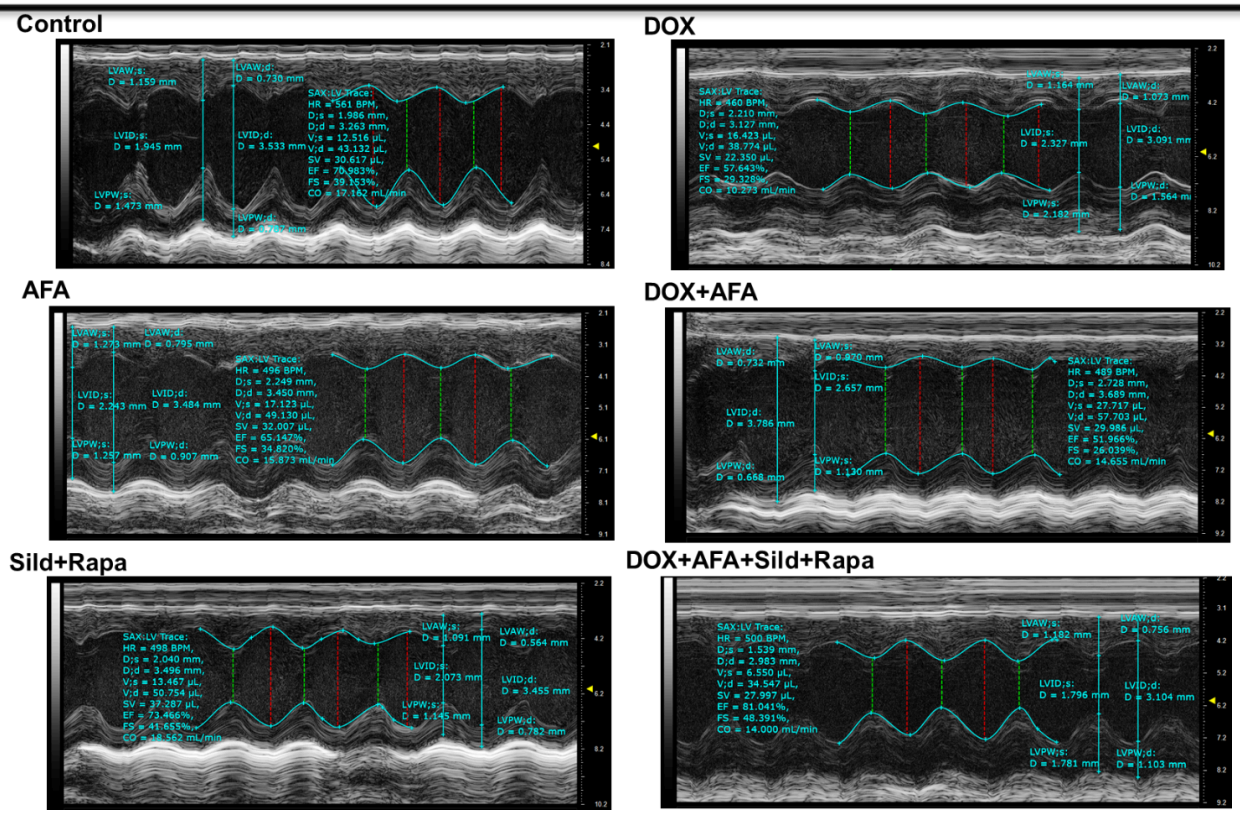

Figure 23: **Effect of Combination Therapy on Left Ventricular Function (Representative M-Mode Imaging)**

Representative M-mode images of echocardiography of female C57/BL6 mice treated with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/ twice a week, for 2 weeks, i.p.) and/or Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks). Echocardiography measuring LVIDd: Left ventricular end-diastolic diameter; LVIDs: Left ventricular end-systolic diameter; Vd: Left ventricular end-diastolic volume; Vs: Left ventricular end-systolic volume; LVPWd (PWDT): Left ventricular end-diastolic posterior wall thickness; LVPWs (PWST): Left ventricular end-systolic posterior wall thickness; LVAWd (AWDT): Left ventricular end-diastolic anterior wall thickness; LVAWs (AWST): Left ventricular end-systolic anterior wall thickness; SV: Stroke volume; EF: Ejection fraction; FS: Fractional shortening; CO: Cardiac Output, HR: Heart Rate.

Upon initial visualization of echocardiography M-mode imaging, the control group displays representative normal posterior and anterior wall thickness and systolic and diastolic

parameters with an LVEF of 70.983% (**Figure 23**). Mice treatment with DOX alone resulted in thinner and flattened anterior and posterior systolic and diastolic thicknesses and a drastic decrease in LVEF of 57.643%. As shown in **Figure 23**, DOX+AFA treatment caused even more loss in anterior and posterior left ventricular thickness and lower LVEF of 61.966%. Alternatively, Sild and Rapa combinatorial effect with DOX restores cardiac structure and function with greater left ventricular thickness and increased LVEF of 81.041% compared to DOX+AFA treatment **(Figure 23**).

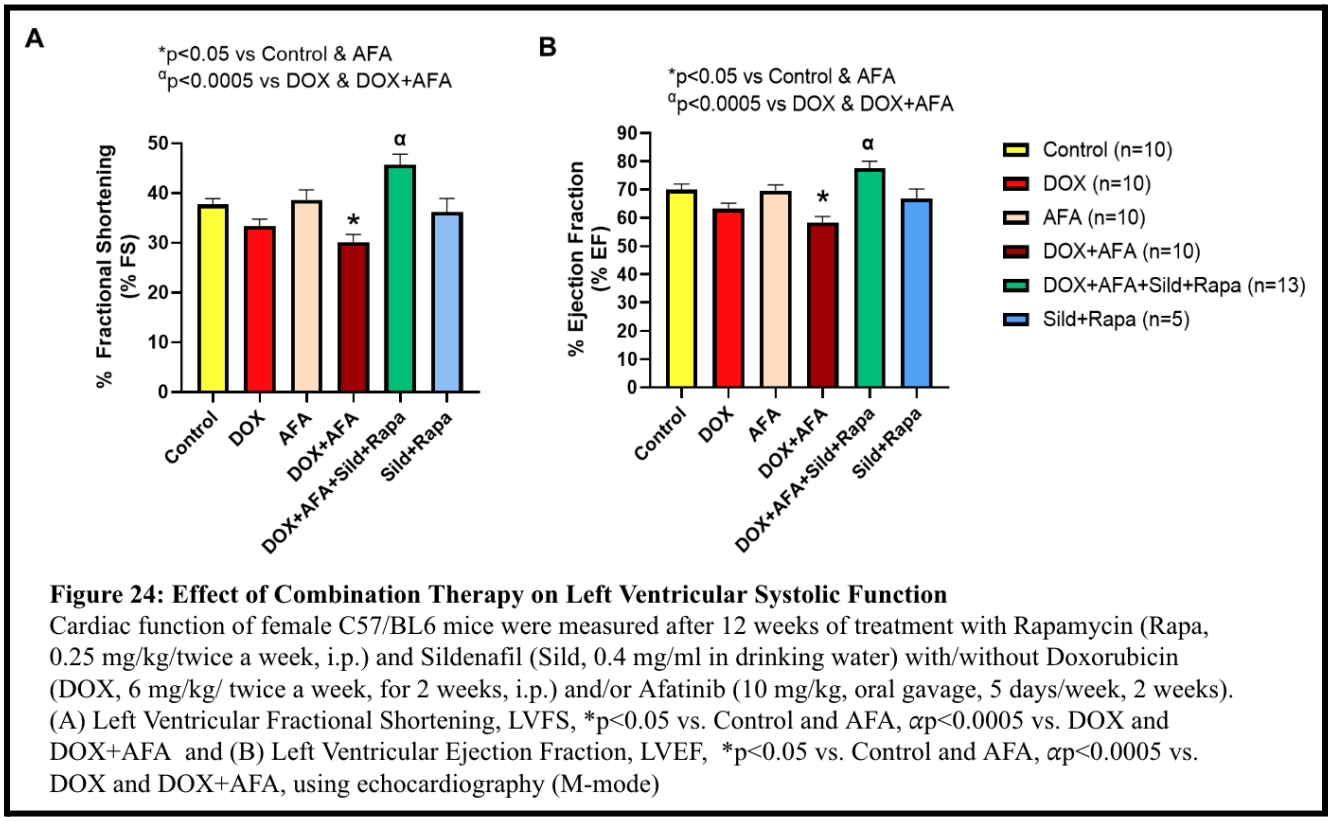

Figure 24: Effect of Combination Therapy on Left Ventricular Systolic Function
Cardiac function of female C57/BL6 mice were measured after 12 weeks of treatment with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/ twice a week, for 2 weeks, i.p.) and/or Afatinib (10 mg/kg, oral gavage, 5 days/week, 2 weeks). (A) Left Ventricular Fractional Shortening, LVFS, *p<0.05 vs. Control and AFA, αp<0.0005 vs. DOX and DOX+AFA and (B) Left Ventricular Ejection Fraction, LVEF, *p<0.05 vs. Control and AFA, αp<0.0005 vs. DOX and DOX+AFA, using echocardiography (M-mode)

Upon analysis of all cardiac functional data, the percentage of LVFS (33%) and LVEF (63%) reduced with DOX treatment as shown in **Figure 24** and were further reduced with combination treatment with DOX and AFA (LVFS: 30%; LVEF: 58%) as compared to the control group (LVFS: 46% and LVEF: 78%). Sild and Rapa additively improved DOX+AFA-induced cardiac dysfunction (ejection fraction and diastolic function) in C57BL/6J mice as compared to DOX alone and DOX+AFA treatment (**Figure 24**)

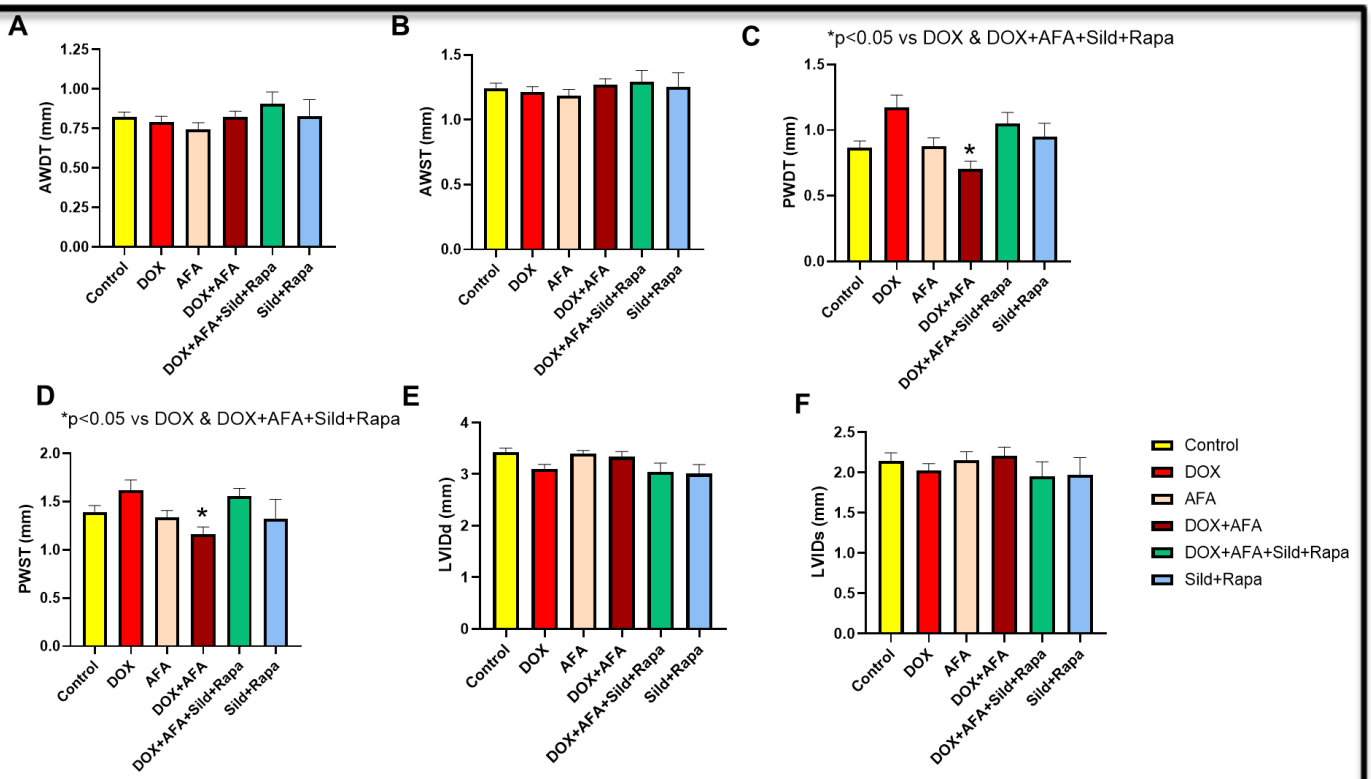

Figure 25: Effect of Combination Therapy on Left Ventricular Cardiac Function

Effect of combination therapy with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/ twice a week, for 2 weeks, i.p.) and/or Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks) on female C57/BL6 mice on (A) AWDT: Anterior wall diastolic thickness; (B) AWST: Anterior wall systolic thickness; (C) PWDT: Posterior wall diastolic thickness, *p<0.05 vs. DOX and DOX+AFA+Sild+Rapa; (D) PWST: Posterior wall systolic thickness, *p<0.05 vs. DOX and DOX+AFA+Sild+Rapa; (E) LVIDd: Left ventricular internal diameter at end diastole; (F) LVIDs: Left ventricular internal diameter at end systole.

There were no significant differences between treatment groups for AWDT, AWST, LVIDd and LVIDs, but DOX+AFA treatment significantly reduced PWDT (0.7 mm) and PWST (1.16 mm) as compared to DOX alone (PWDT:1.17 mm and PWST:1.62 mm) and DOX+AFA+Sild+Rapa groups (PWDT:1.05 mm and PWST:1.56 mm) (**Figure 25**).

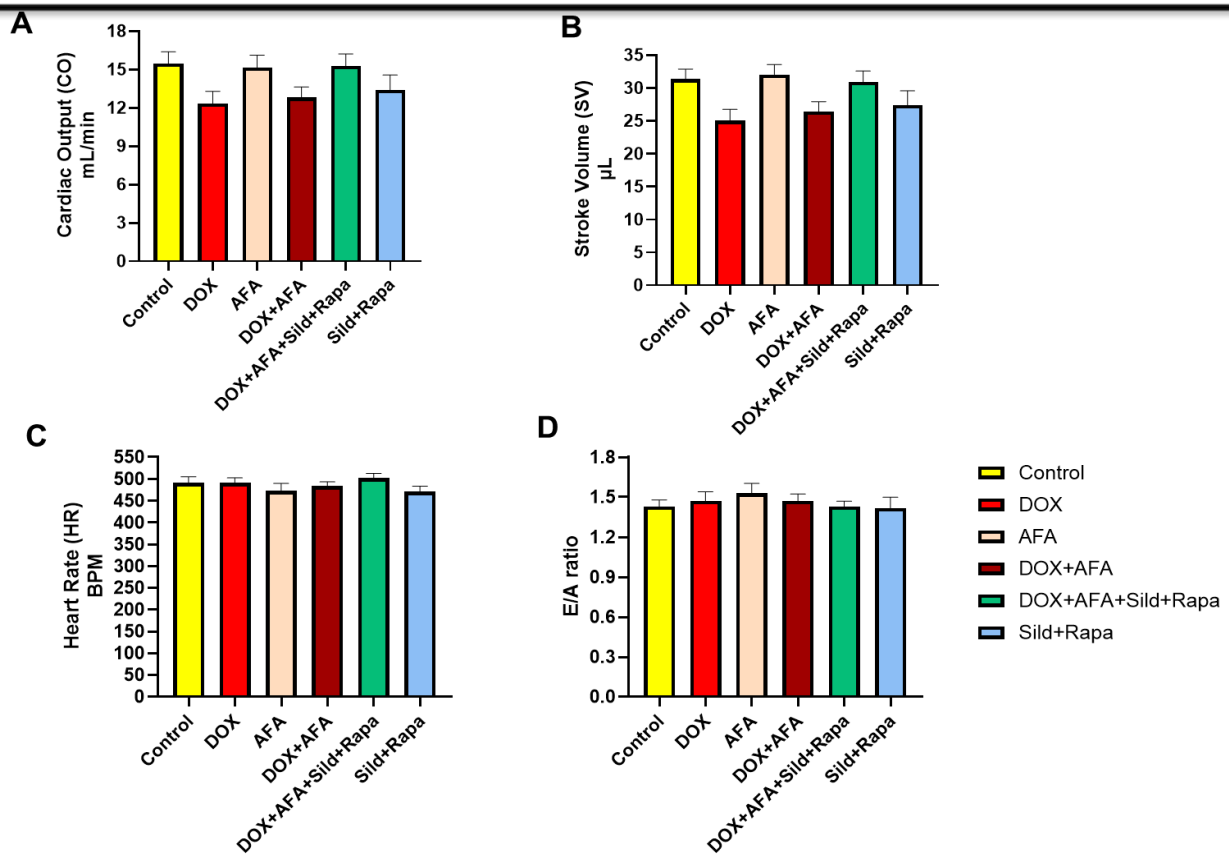

Figure 26: Effect of Combination Therapy on Diastolic and Systolic Function

Effect of **c**ombination therapy with Rapamycin (Rapa, 0.25 mg/kg/twice a week, i.p.) and Sildenafil (Sild, 0.4 mg/ml in drinking water) with/without Doxorubicin (DOX, 6 mg/kg/ twice a week, for 2 weeks, i.p.) and/or Afatinib (AFA, 10 mg/kg, oral gavage, 5 days/week, 2 weeks) on female C57/BL6 mice on (A) CO: Cardiac Output; (B) SV: Stroke volume; (C) HR: Heart Rate; (D) E/A ratio.

Cardiac Output (CO) and stroke volume (SV) were reduced in mice treated with DOX alone and DOX+AFA as compared to control, but not significantly (**Figure 26**). Sild+Rapa in combination with DOX+AFA improved both CO and SV, recovered to control levels (**Figure 26**). Additionally, heart rate and E/A ratio were not significantly changed between treatment groups (**Figure 26**).

DISCUSSION

Firstly, breast cancer remains a predominant global health concern, with increasing incidence rates projected due to demographic shifts and lifestyle changes (**Arnold et al, 2022**). The standard treatment protocols often involve DOX, a potent anthracycline chemotherapy known for its efficacy in breast cancer among the various cancer types. However, its clinical utility is limited by severe cardiotoxic effects, which manifest as acute and chronic cardiac complications, leading to significant morbidity and mortality among cancer patients and survivors (**Johnson-Arbor & Dubey, 2023; Gabani et al, 2021; Kong et al, 2022**). Thus, more research is required to lessen the adverse effects without compromising the therapeutic efficacy of DOX in combating cancer cells. The proposed strategy of combining PDE5 inhibitors (Sild) and mTOR inhibitors (Rapa) alongside DOX presents a novel approach to address these challenges. The findings underscore the therapeutic potential of combining Sild and Rapa with conventional chemotherapy (DOX) in enhancing treatment efficacy across different breast cancer subtypes, while also highlighting a potential cardioprotective role in mitigating chemotherapy and immunotherapy-induced cardiotoxicity.

Enhanced Chemotherapy Efficacy

The results demonstrate that the combination of Sild and Rapa significantly improves the efficacy of doxorubicin (DOX) in killing TNBC cells across multiple models, including MDA-MB-231 and 4T1 breast cancer cells. Combination of Sild and Rapa with DOX suppressed mTOR activity, specifically mTORc1 as indicated by reduction of p-mTOR as well as pS6 levels in both MDA-MB-231 and 4T1 cells. This combination strategy also induced DOX-induced apoptosis of TNBC cells by suppressing antiapoptotic protein Bcl2 level, but increasing cleaved-PARP level. This aligns with previous research showing that Rapa enhances DOX efficacy by inhibiting the mTOR pathway, which is crucial for cell survival and proliferation (**Li et al. 2019**). Additionally, Sild has been shown to enhance the effectiveness of chemotherapy through modulation of the cGMP pathway (**Li et al., 2023**). The combined approach in our study offers a robust strategy for overcoming resistance mechanisms and improving therapeutic outcomes.

ErbB2 Inhibition

Incorporating an ErbB2 inhibitor, AG825 into the treatment regimen allowed us to assess the combined effects on ErbB2-positive breast cancer cells, while mitigating toxicity to cardiomyocytes. Previous research has established that ErbB2 overexpression contributes to both cancer progression and resistance to therapy (**Zhang et al., 2021**). The addition of AG825 enhances the anti-cancer effects of DOX, suggesting a multi-faceted approach to targeting ErbB2-positive cancers. Combination of Sild and Rapa potentiates therapeutic efficacy of DOX and AG825 in ErbB2-positive breast cancer cells by significantly enhancing cell death and reducing cell viability. This combination treatment also inhibits mTOR activity by suppressing both mTOR complexes, as depicted by reduction of pS6 (marker of mTORc1) and pAKT (marker of mTORc2). Sild+Rapa in combination with DOX+AG825 induces cleaved PARP level as well as Bax protein, the markers of apoptosis in ErbB2 positive breast cancer cells. This additive strategy may provide a more comprehensive treatment plan for patients with ErbB2-positive tumors.

Cardioprotection

Cardio-oncology has emerged as a crucial field due to the dual challenge of effectively treating cancer while minimizing cardiotoxicity associated with chemotherapeutic agents. Recent advances in combination therapies aim to address both concerns simultaneously. Our previous study demonstrated that Sild enhances chemotherapeutic efficacy of DOX in prostate cancer, but concurrently provides cardioprotection (**Das et al. 2010**). The present study advances the field by demonstrating that targeted combination therapies can achieve both goals, potentially transforming treatment paradigms for patients undergoing chemotherapy. The cardioprotective effects of Sild and Rapa were assessed in AC16 cardiomyocytes and a murine model. Previous studies have suggested that Sild mitigates DOX-induced cardiotoxicity by promoting nitric oxide signaling and reducing oxidative stress (**Pushkar et al., 2021**). Rapa, known for its anti-inflammatory and anti-fibrotic properties, also contributes to cardioprotection by modulating autophagy and reducing myocardial damage (**Chen et al., 2022**). Our results show that Sild and Rapa co-treatment protects cardiomyocytes against DOX and AG825-induced cell toxicities. Combination treatment mitigates mTOR activity in cardiomyocytes with reduction of apoptotic protein BAX as compared to DOX+AG825 treatment. The present *in vivo* murine study shows

cardiac dysfunction following combination of chemotherapy (with DOX) and immunotherapy (with AFA). Co-treatment with Sild and Rapa restores cardiac function of mice subjected to chemo and immunotherapy, supporting the cardioprotective benefit of combination treatment.

Unique Aspects of the Study

While individual components like Sild, Rapa, and AFA have been studied, their combined effects on both enhancing chemotherapy and providing cardioprotection are less explored. Our study integrates these components in a novel way, addressing two critical issues—cancer efficacy and cardiotoxicity simultaneously. By utilizing multiple breast cancer cell lines (MDA-MB-231, 4T1, SKBr3) and human cardiomyocytes, along with a murine model, our study provides a comprehensive evaluation of the therapeutic and protective effects of the combination therapy. This multi-model approach strengthens the validity and applicability of the findings across different biological systems.

Implications for Future Research and Clinical Practice

The combination of Sild and Rapa with Afatinib offers a promising strategy for improving chemotherapy outcomes while protecting the heart. Future clinical trials could explore this combination therapy in diverse patient populations to validate its efficacy and safety. As cardiotoxicity remains a major concern in cancer treatment, our study highlights a potential avenue for reducing such adverse effects. Integrating cardioprotective agents like Sild and Rapa into chemotherapy regimens may become a standard practice to enhance patient quality of life. The unique combination approach paves the way for further exploration of other drug combinations and their potential additive effects in cardio-oncology. This could lead to the development of new therapeutic strategies that address both cancer and cardiovascular health. In summary, this study presents a significant advancement in the field of cardio-oncology by demonstrating how combination therapies can enhance chemotherapy efficacy and provide cardioprotection. The integration of Sild, Rapa, and Afatinib in our research offers a novel approach with the potential to improve patient outcomes and set the stage for future clinical innovations.

LIMITATIONS

We have proposed one concentration of each drug for combination therapy based on our previous studies (**Das et al. 2005, 2010, 2012**). Testing for optimal Rapa and Sild concentrations in combination with DOX can ensure maximal cytotoxic effects and cardioprotection. In the current study, we used AC-16, which is proliferating human cardiomyocyte cell lines derived from non-proliferating primary cultures of adult ventricular heart tissue. However, this proliferative cardiac cell line has numerous limitations including their capability of cell division, loss of specific cardiac features and resistance to hypoxic injury, that cause low translation impact due to the low resemblance of AC-16 cells to mature adult cardiac tissue (**Onódi et al. 2021**). In the future, we will conduct cardiotoxic experiments using primary cardiomyocytes isolated from adult female mice and using human iPSC (Induced pluripotent stem cells)-derived cardiomyocytes. Although these strategies are costly, these *in vitro* strategies can confirm the protective effects of combination therapy on the heart.

Additionally, co-treatment assays can potentially cause experimental dynamic issues. This can be mitigated by standardizing time for cell death of co-cultured cardiomyocytes by propidium iodide staining followed by microscopic observations of cell monolayers.

For *in vivo* cardiotoxicity study, due to the significant reduction of body weight and survival of female mice after DOX and DOX+AFA treatment, mice had been sacrificed. Therefore, several parameters of cardiac function are not significantly different between groups. Additional experiment with longer time of DOX+AFA treatment is warranted to confirm the beneficial effect of Sild+Rapa against DOX+AFA-induced cardiotoxicities.

FUTURE WORK

Based on preliminary results, the hypothesis is supported. The interaction between DOX, Sild, and Rapa not only enhances the anti-cancer potential, but also addresses concerns of DOX related cardiotoxicity, making it a standout and highly effective combination in the landscape of cancer therapy. To better understand the associated pathways, I will examine further to identify novel signaling pathways associated with combination treatment with Sild and Rapa which could enhance cytotoxicity DOX in cancer cells, but protect cardiac cells against DOX-induced

toxicities. An additional experiment includes RNA sequencing using next-generation sequencing to isolate and quantify RNA profiles following treatment of each group for all tested cell lines.

We will examine the cardiac apoptosis and fibrosis in the hearts of DOX + Afatinib treated female C57 mice and compare these with Sild+ Rapa co-treated hearts. We will identify the mechanisms associated with Sild+ Rapa-induced cardioprotection against DOX + Afatinib-induced toxicities. Future research with tumor bearing mice will be conducted to confirm the enhanced chemo/immunotherapeutic efficacy with combination treatment of Sild and Rapa, and its cardioprotective effect. Additional studies are warranted to translate these promising preclinical results into clinical settings for improved therapeutic outcomes in breast cancer patients.

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