PROBUCOL IS CARDIOPROTECTIVE AGAINST ANTHRACYCLINE AND TRASTUZUMAB MEDIATED CARDIOTOXICITY

BY

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Abstract

Background: In breast cancer patients, approximately 20-30% of cases have overexpression of the Human Epidermal Growth Factor-2 receptor (HER2/ErbB2) which is an aggressive form of the malignancy, often resistant to standard anthracycline-based chemotherapy. Trastuzumab, a monoclonal antibody, is used for the treatment of HER2/ErbB2 breast cancer. However, the administration of Doxorubicin (D) and Trastuzumab is associated with an increased risk of cardiotoxicity. Although oxidative stress plays a key role in the pathogenesis of Doxorubicin-induced cardiac dysfunction, it's role in the combined setting of Doxorubicin and Trastuzumab mediated cardiotoxicity remains unknown.

Objective: The aim of the study was to determine if the antioxidant Probucol would be useful in attenuating this drug induced cardiotoxicity from Doxorubicin and Trastuzumab.

Methods: In an acute murine model of chemotherapy induced cardiomyopathy, wild-type C57Bl/6 mice received one of the following regimens: (1) control; (2) Doxorubicin (D); (3) Trastuzumab (T); (4) Doxorubicin+Trastuzumab (D+T); (5) Probucol (P); (6) Probucol+Doxorubicin (P+D); (7) Probucol+Trastuzumab (P+T); or (8) Probucol+Doxorubicin+Trastuzumab (P+D+T). Serial murine echocardiography with tissue Doppler imaging was performed daily for 10 days. At 10 days post-treatment, the hearts and livers were removed for histopathologic and Western blot analyses.

Results: Mice treated with prophylactic Probucol demonstrated minimal cardiotoxicity compared with those treated with Doxorubicin and Trastuzumab. Left ventricular (LV) cavity dimensions and LV systolic parameters were preserved in mice prophylactically treated with Probucol following the administration of Trastuzumab and Doxorubicin. The survival rate was 27% at day 3 of the experiment in the D+T group which significantly improved to 82% in mice receiving P+D+T. Histological findings were consistent with increased vacuolization and myofibrilar degeneration in the placebo groups which was attenuated in the probucol groups. Trastuzumab and Doxorubicin caused an increase in Bax/Bcl-XL which was about 3 fold higher in the combined D+T group with a density ratio of 21. Probucol significantly blunted this increase in proapoptotic/antiapoptotic protein expression where the density ratio was 3.0 in the combined D+T group.

Conclusion: The synergistic cardiotoxic effects of Doxorubicin and Trastuzumab is partially attenuated by the prophylactic administration of the antioxidant Probucol.

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List of Abbreviations

ACEI	Angiotensin-Converting Enzyme inhibitors
ARB	Angiotensin Receptor Blocker
β-blocker	Beta-Blocker
BCIRG 006	Breast Cancer International Research Group 006 data trial
Bcl-XL	B cell lymphoma 2 family
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CAD	Coronary Artery Disease
CHF	Congestive Heart Failure
CISH	Chromogenic *in situ* hybridization
CKO	Conditional knockout
CMR	Cardiac MRI
CRCD	Chemotherapy related dysfunction
D	Doxorubicin
D+T	Doxorubicin plus Trastuzumab
DCIS	Ductal carcinoma *in situ*
DMSO	Dimethysulfoxide
EF	Ejection Fraction
EGFR/ErbB1	Human Epidermal Growth Factor Receptor-1
EGFR/ErbB2	Human Epidermal Growth Factor Receptor-2
Erb-hcAb	anti-ErbB2-compact antibody
Erb-hrRNase	Erbicin-human-RNase
ErbB(1-4)	Human Epidermal Growth Factor Receptor-(1-4)
ErbB3	Human Epidermal Growth Factor Receptor-3
ErbB3	Human Epidermal Growth Factor Receptor-4
ER	Estrogen receptor
FinHer	Finnish Herceptin trials
FISH	Fluorescence in situ hybridization
FS	Fractional Shortening
gp130	Glycoprotein 130
HER2/ErbB2	Human Epidermal Growth Factor Receptor-2
HERA	Herceptin Adjuvant trial
Herceptin	Trastuzumab
HR	Heart Rate
IL	Interleukin

IVS	Intraventricular septum
LV	Left Ventricle
LVEDD	LV end-diastolic diameter
LVEDS	LV end-systolic diameter
LVEF	Left ventricular ejection fraction
MAPK	Mitogen-activated protein (MAP) kinases
MRI	Magnetic Resonance Imaging
MUGA	Multiple-gated acquisition
N-acetylcysteine	N-acetyl-L-cysteine
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NRG	Neuroregulin
OD	Optical Density (absorbance)
P	Probucol
P+D	Probucol plus Doxorubicin
P+D+T	Probucol+Doxorubicin+Trastuzumab
P+T	Probucol plus Trastuzumab
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered solution
PI3K	Phosphatidylinositol 3-kinases
PWT	Posterior wall thickness
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxidative species
RT3D TTE	Real-time three-dimensional transthoracic echocardiography
SOD	Superoxide dismutase
SR	Radial strain rate
T	Trastuzumab
T-Tubule	Transverse tubule
TDI	Tissue Doppler imaging
TP53	Tumor protein 53
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
V_{ENDO}	Peak systolic endocardial velocity

Chapter 1 Review of the Literature

1.1 Breast cancer: Introduction

Breast cancer is the most prevalent form of malignant disease in women with an estimated 1.05 million cases worldwide.[1] In North America, it is estimated that over 250 000 women will be newly diagnosed with breast cancer in 2010, leading to 45,000 deaths.[2, 3] Treatment advances for this major public health problem continue to focus on aggressive screening procedures for early detection and specialized treatment regimens.

Advances in imaging have decreased the morbidity and mortality associated with breast cancer due to early detection. Some of these advances in early detection, including full-field digital mammography, magnetic resonance imaging, and positron-emission tomography/mammography.[4]

The treatment for breast cancer is multifaceted, involving a combination of surgery, radiation therapy and chemotherapy. Mastectomy is often the first line approach for breast cancer, where the procedure varies depending on adjacent nodes affected. Approaches range from local node specific lymphadenectomy to radical mastectomy where all of the breast tissue is removed.[5, 6] Radiation therapy is an integral component of early stage breast cancer treatment as it is effective with both surgery and chemotherapy.[7] It has been shown to reduce the risk of local recurrence and is particularly responsive in the adjuvant use, which is applied after

initial chemotherapy. Chemotherapeutic agents generally include cyclophospamide and anthracyclines, including either Doxorubicin or Epirubicin. Finally, in those patients who express estrogen or progesterone positive receptors, adjuvant hormonal therapy is warranted.[8]

1.2 Anthracyclines: Doxorubicin anti-tumor mechanisms

Doxorubicin (Adriamycin) is currently used as a potent anti-tumor chemotherapy drug in the first line treatment of breast cancer. Doxorubicin is a member of the anthracycline family of chemotherapy drugs that was originally isolated from soil microbes. The microbe *Streptomyces* was found to produce the antibiotic Daunomycin in 1957 which demonstrated the ability to inhibit cell division.[9] Doxorubicin differs from Duanorubicin by a single hydroxylated 14th carbon (Figure 1). Doxorubicin is a cleavable complex-forming topoisomerase II inhibitor used for cancer since 1963[10-12] and acts by inhibiting mammalian cell DNA, RNA synthesis and arresting uncontrolled tumor growth.[13, 14] This is accomplished via readily binding to nucleic acids and specifically targeting topoisomerase-II alpha (topo-IIa*)*. Topo-IIa is mechanistically a target for a number of anti-neoplasmic agents in addition to Doxorubicin.[15, 16] DNA topoisomerases execute a vital role in DNA replication and in the segregation of daughter chromosomes by catalyzing the interconversion of topological DNA isomers, which is a modification of linking numbers.[17] Specifically, the type II enzymes function by transferring a DNA segment through a transient double

strand break in DNA.[17] Doxorubicin forms a cleavable complex, stabilizing the topo-IIa-DNA cleavable complex by intercalation, stimulating double-stranded DNA scission at specific sites.[18]

Unlike the topoisomerase mechanism proposed above for Doxorubicin, antitumor effects induced by reactive oxidative species (ROS) indicate a non-protein-associated DNA strand breaks. A sequence of reactions known as redox cycling leads to the generation of ROS. Doxorubicin promotes the production of ROS *via* the binding of iron and formation of an iron complex.[19] A semiquinone free radical is formed from an electron reduction of ring B. Although stable under anaerobic conditions, the unpaired electrons are donated to oxygen under aerobic conditions and a superoxide radical is formed. Inhibition of DNA strand damage by preventing Poly ADP-ribose polymerase (PARP) activation *via* superoxide dismutase (SOD), dimethysulfoxide (DMSO), catalase and antioxidants suggests that free radical formation indeed plays a role in the antitumor efficacy of Doxorubicin.

1.3 Anthracyclines: Anthracycline-induced cardiotoxicity

As early as 1967, observations of adverse cardiac effects were observed with Doxorubicin. Acute and chronic cardiotoxic effects were apparent including electrocardiographic T-wave inversions, damaged cardiac ganglia, myocyte death and heart failure.[20]

Cardiotoxic side effects affect the long-term health status of breast cancer survivors.[21, 22] Common risk factors for developing Doxorubicin mediated cardiac dysfunction include a cumulative dose that exceeds 400 mg/m^2, age greater than 70 years, left chest radiotherapy, and a past history of hypertension.[23-25] Identifying risk factors may predict which patients are more susceptible to cardiotoxicity with routine cardiac assessment is critical in this setting.[26] As the cytotoxic properties of this drug are not specific to tumor cells, myocyte cells within the myocardium are also affected. Often this prompts myocardial damage and apoptosis leading to congestive heart failure (CHF).[21, 22] The degree of Doxorubicin-induced cardiomyopathy is proportional to the cumulative dose of drug administered. As such, the chemotherapeutic treatment of breast cancer malignancies is essentially limited by a conundrum of targeting to achieve maximum anti-tumor effectiveness while maintaining the lowest possible cumulative dose to prevent cardiotoxicity.

1.4 Doxorubicin-induced cardiotoxicity: Oxidative stress

In Doxorubicin-induced cardiomyopathy, several physiological mechanisms have been proposed and debated upon. It is generally agreed upon that oxidative stress plays a key role.[23] As ROS is implicated as an underlying mechanism for Doxorubicin's anti-tumor effects, it was also suggested as a mechanism for Doxorubicin-induced cardiotoxicity based upon the following observations: i) a concentration-related increase in superoxide anion and hydrogen peroxide in heart

sarcomeres, mitochondria and cytosol with anthracycline treatment; and ii) degeneration of heart mitochondria and sarcoplasmic reticulum is characteristic in anthracycline mediated cardiotoxicity.[27, 28] The generation of ROS may be attributed to the interaction of Doxorubicin's quinone ring with mitochondrial enzymes within cardiomyocytes.[25, 29] This is believed to lead to an increase in ROS levels and subsequent DNA damage and/or lipid peroxidation of cardiomyocytes.[23] It is this ROS induced oxidative stress acting upon the myocardial cells that is suggested to cause Doxorubicin-induced cardiomyopathy.

Altered expression of proapoptotic and antiapoptotic protein is implicated in the pathology of cardiomyopathies including hypertrophic[30] and dilated cardiomyopathy.[31-33] In dilated cardiomyopathy, apoptosis is associated with an upregulation of proapoptotic proteins including Bax, caspase 3 and p53.[34] Similarly, there is a downregulation of antiapoptotic proteins including the B cell lymphoma 2 family (Bcl-XL) which are equally important in the regulation of cardiomyocyte apoptosis.[34] The ratio of Bax/Bcl-XL has been implicated in the pathogenesis of Doxorubicin-induced cardiomyopathy as higher levels of proapoptotic to anti-apoptotic proteins signal cardiomyocyte apoptosis.[35] In the aforementioned studies linking Doxorubicin treatment to oxidative stress and ROS formation, apoptosis has been suggested as the underlying mechanism for Doxorubicin-mediated cardiomyopathy.[23, 35-48] This is supported by an increase in proapoptotic Bax protein expression and decreased antiapoptotic Bcl-XL expression along with histological confirmation of myofibular loss.[40, 49-53]

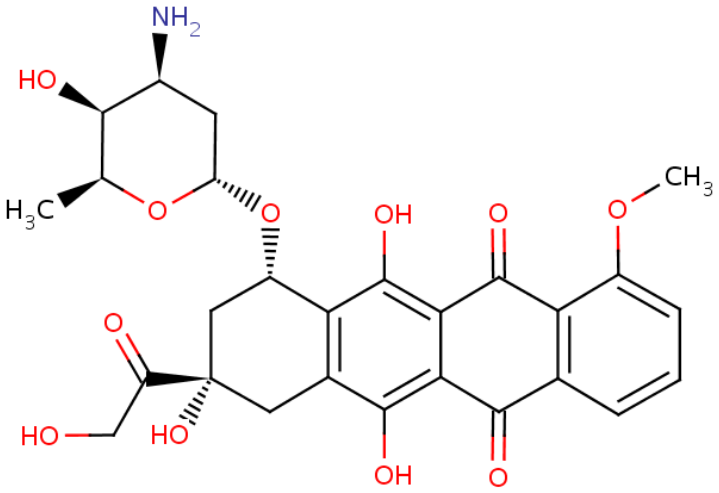

Figure 1. Chemical structure of Doxorubicin: 7S,9S-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione.[54]

1.5 Oncogenes and their role in carcinogenesis

Recent advances in the management of breast cancer patients have carefully considered predisposing genetic factors underlying both its development and its progression. With a strong correlation between family history and the onset of breast cancer,[55] the BRCA1, BRCA2 and TP53 genes are most frequently involved.[56] Women who carry mutations among BRCA1 and BRCA2 are at a three to four times increased lifetime risk for developing breast cancer.[56] In women who develop breast cancer, palpability, larger neoplasm size, nuclear grade (>2), metastasis to adjacent axillary lymph nodes, estrogen receptor and HER2 status are poor prognostic factors.

1.6 Oncogenes and their role in carcinogenesis: Estrogen receptors

Mutations among genes encoding for estrogen receptors (ER) have shown a strong positive correlation with the occurrence of breast cancer. ERα, located on chromosome 6q25.1,[57] has been well described in the etiology of breast cancer. Women with overexpression of ERα have an elevated risk of developing breast cancer by a factor of 1.5.[57] Treatment regimens that target ER receptors have proven greater efficacy than standard broad spectrum treatment. In this setting, the use of tamoxifen as an ER receptor inhibitor for chemoendocrine therapy, improves the efficacy of treatment compared to non-ER targeting therapy. One study showed that a regimen consisting of 6 months of standard adjuvant anthracyclines followed by 5-years tamoxifen therapy reduces the average breast cancer death rate by 31% for women between all age groups in this clinical setting.[58]

1.7 Human Epidermal Growth Factor Receptors: ErbB2 signaling pathways

ErbB receptors are a group of tyrosine kinases belonging to the epidermal growth factor receptor family and are responsible for mediating cell growth, differentiation and survival.[59, 60] In general, signaling via these receptors involves crosstalk and a number of signaling events arranged in a complex and interdependent cellular network.[60] However, the exact mechanisms and signal pathways remain poorly understood, especially for the ErbB2 isoform.[60] Four isoforms are expressed including EGFR/ErbB1, HER2/ErbB2, ErbB3, and ErbB4. These isoforms are expressed within cardiomyocytes of the fetal and adult heart.

Each of these receptors has the ability to act as a co-receptor in neuroregulin (NRG) signaling.[61, 62] Each of the ErbB family receptors exhibits the ability to couple to a unique intracellular signaling pathway. ErbB2 does not directly bind to ligands including NRG, but instead heterodimerizes with adjacent ErbB receptors for initiating downstream cell signals of cell survival and proliferation (Figure 1).[61, 62]

ErbB2, also commonly referred to as HER2 or *c-neu,* is of specific importance in the setting of breast cancer. This receptor's extracellular domain is functionally ligand-independent. The receptor is transactivated by G protein-coupled receptors, while it is regulated by intracellular signals. G-protein-coupled receptors have a positive effect on ErbB signaling though the activation of matrix metalloproteinases and through the indirect activation of Src.[59, 63, 64] Upon stimulation by a ligand such as cytokine interleukin (IL)-6 or NRG, the receptor dimerizes with adjacent ErbB receptors ErbB1-4. Once dimerized with an adjacent receptor, ErbB2 signals downsteam cell proliferation and survival via PI3K/Akt and ras-Raf-MAPK pathways respectively. In ErbB2 overexpression, this results in increased intensity of influence on survival and proliferation signals.[65] Counteracting the binding affinity of ErbB2 to ligands are PKC activating growth factors and hormones which decrease tyrosine phosphorylation. Intermediate signal mediating sequences of EGF-R are required for ErbB2 activation and transmodulation.[66] This suggests that overexpression of ErbB2 affects the degree of crosstalk between adjacent receptors and other signaling pathways.[64] A study

on ErbB2 expression vectors showed that ErbB2 and EGFRs couple with mitogenic signaling pathways.[67] This has direct implications in cellular oxidative stress and apoptotic pathways which are a key component of anti-ErbB2-induced cardiomyopathy.

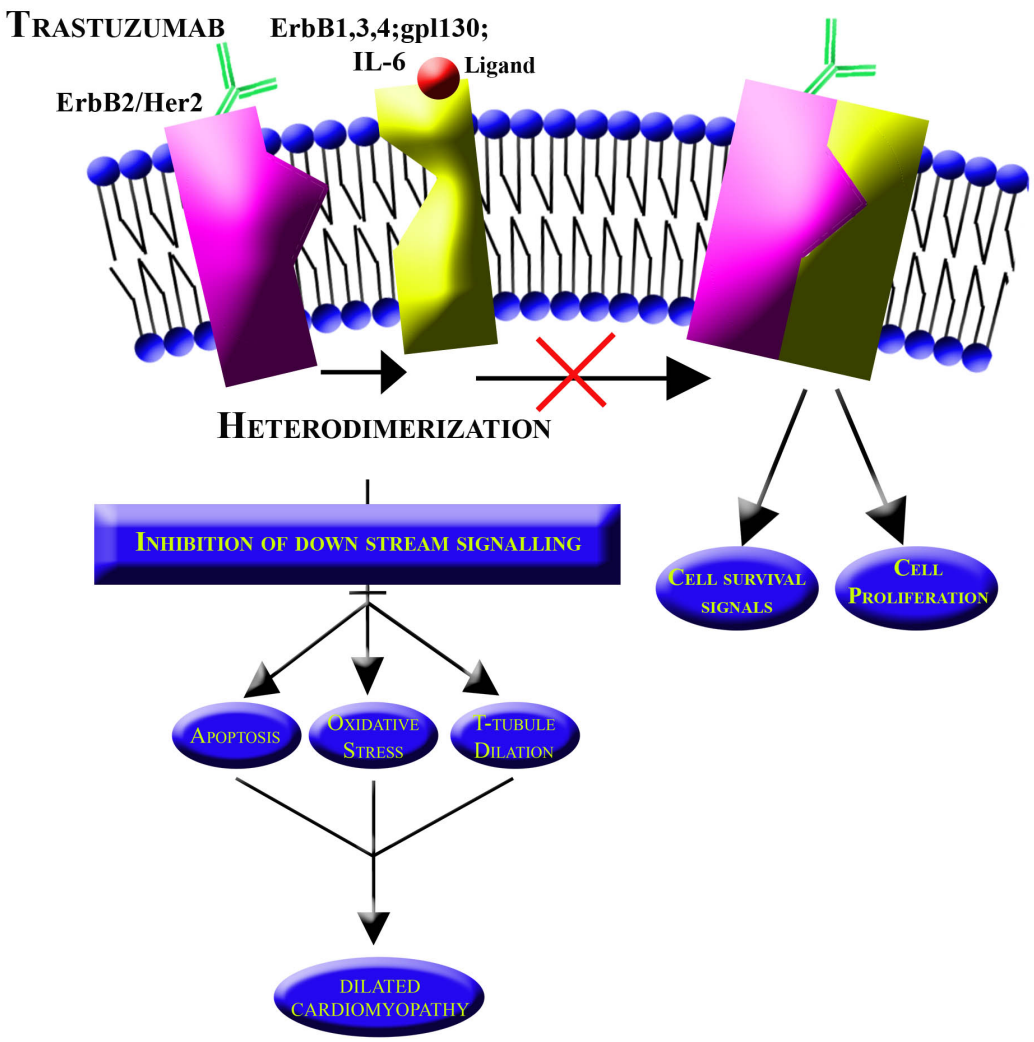

Figure 2. Normal heterodimerization of the human epidermal growth factor receptor 2 (HER2) with adjacent receptors (ErbB1, ErbB3, ErbB4, gp130 and IL-6). Trastuzumab binds to HER2, inhibiting downstream signalling with subsequent development of a dilated cardiomyopathy. (originally published in Exp Clin Cardiol 2009 Walker J et al:14(3):e62-7)

1.8 HER2/ErbB2 in Breast Cancer

It has been well established that overexpression of the human epidermal growth factor receptor (HER/ErbB) family of transmembrane tyrosine kinase receptors is present in numerous forms of carcinoma.[68-70] Approximately 25-30% of breast cancer patients overexpress the HER2/ErbB2 receptors.[71, 72] Specific forms of breast cancer, including ductal carcinoma *in situ* (DCIS) significant overexpress ErbB2 in approximately 90% of cases.[73]

Overexpression of HER2 generally confers a poor prognosis as it promotes aggressive tumor growth and hinders the effectiveness of standard chemotherapy.[74] ErbB2 positive tumors arise from amplification of the gene responsible for transcribing the ErbB2 receptor. An increased number of ErbB2 receptors on the cell surface accelerates the rate of cell division and multiplication ensuing an aggressive malignancy.[75] These cells display hyperactivity of their cell cycle machinery and become resistant to standard chemotherapy and radiotherapy-induced apoptosis.[73, 76] As a result of this aggressiveness and resistance to cytotoxic agents, it is clinically accepted that a high ErbB2 expression predicts lower survival outcomes.[77]

Early detection of ErbB2 overexpression following the diagnosis of breast cancer is critical prior to assigning treatment regimens as a positive finding necessitates aggressive treatment.[74] Overexpression of HER2 is detected using either cell-

based chromogenic *in situ* hybridization (CISH) or fluorescence *in situ* hybridization (FISH).[78, 79]

Detection of HER2 overexpression in breast cancer patients yields several clinically useful applications including, but not limited to, predicting prognosis and response to drug regimens including endocrine therapy, tamoxifen, chemotherapeutic drugs, and anti-HER2 drugs.[80]

1.9 HER2/ErbB2: The inhibitory effect of Trastuzumab

Trastuzumab (Herceptin) is recommended for treatment of breast cancer patients with overexpression of HER2/ErbB2 (Genentech, Inc, South San Francisco, CA 2000).[72] Trastuzumab is a monoclonal antibody that directly recognizes and inhibits HER2/ErbB2 receptors.[81] Specifically, it effectively binds to extracellular HER2/ErbB2 and interrupts its signaling pathway. Interruption of ErbB2 subsequently inhibits critical downstream survival and proliferation signaling pathways such as ras-Raf-MAPK and PI3K/Akt.[64, 72] Additionally, Trastuzumab downregulates HER2/ErbB2 expression through enhancing internalization and degradation.[77, 82] Antibodies specific for HER2/ErbB2, including Trastuzumab, have been shown to elevate cyclin-dependent kinase inhibitors $P27^{Kip1}$ and the Rb-related protein p130. Sliwkowski *et al.* (1999) outlined that this induction of the $P27^{Kip1}$ reduces the number of cells undergoing S-phase.[60] Additiontally, Trastuzumab's binding to HER2/ErbB2 overexpressing tumor cells is associated

with immune activation and requirement of effectors cells.[80] Thus, Trastuzumab acts by inhibiting HER2/ErbB2 signals that promote cell survival and uncontrolled cell growth cycles.

1.10 The clinical use of Trastuzumab

The clinical use of Trastuzumab varies between adjuvant therapy in breast cancer patients and its use in metastatic disease.[71, 72, 83, 84] In the adjuvant setting of breast cancer, where anthracycline based compounds are used, Trastuzumab is generally administered following completion of chemotherapy, with a loading dose of 8 mg/kg and a weekly maintenance dose of 6 mg/kg for one year.[85] The use of Trastuzumab in the metastatic setting, is administered with loading and maintenance doses of 4 mg/kg and 2 mg/kg, respectively, given every three weeks.[86] As first-line treatment, Trastuzumab has been added to paclitaxel or anthracyclines along with cyclophosphamide. The use of Trastuzumab has also been evaluated for a number of other chemotherapeutic regimens including capecitabine, cisplatin, gemcitabine, vinorelbine and others.[87] In situations where patients have risk factors significant for cardiotoxicity, Trastuzumab has been given as monotherapy. Once administered, Trastuzumab has a mean half life of 28.5 days. However, the metabolic pathways for eliminating the drug remain undefined.[71, 72, 88, 89]

1.11 The clinical use of Trastuzumab: Adjuvant setting

In the adjuvant setting of breast cancer, Trastuzumab improves patient survival and reduces rates of reoccurrence and progression. Trastuzumab use has consistently shown a 50% reduction in rates of breast cancer reoccurrence.[90] A number of clinical trials have evaluated the efficacy of Trastuzumab when it is incorporated into a number of chemotherapeutic treatment regimens. Four multicentre randomized controlled trials have studied the use of Trastuzumab in the adjuvant setting including the Herceptin Adjuvant trial (HERA),[91] National Surgical Adjuvant Breast and Bowel Project trial B-31 trial, North Central Cancer Treatment Group trial N983,[92, 93] and the Breast Cancer International Research Group 006 data (BCIRG 006) trial.[94] The maximum cumulative dose of Doxorubicin in these landmark trials did not exceed 360 mg/m^2 and Epirubicin did not exceed 720 mg/m^2. Overall findings from these trials revealed that in those breast cancer patients who had received at least four cycles of a neo-adjuvant chemotherapy followed by one year of Trastuzumab therapy, there was a 34-41% reduction in the reoccurrence of malignancies along with an improvement in overall disease free survival.[95-97] Overall, these landmark trials have lead to a universally accepted practice that the addition of Trastuzumab to adjuvant anthracycline and taxane based chemotherapy regimens improves patient survival and lowers the risk of breast cancer reoccurrence.

1.12 The clinical use of Trastuzumab: Metastatic setting

Ten percent of breast cancer patients present with metastatic malignancies.[88] Approximately 35-45% of breast cancer metastatic tumours have been found to over- express HER2. The primary therapeutic outcome of metastatic breast cancer chemotherapy is palliative. The use of numerous agents have been evaluated, including anthracyclines, capecitabine, docetaxel, gemcitabine, paclitaxel and vinorelbine, which have all been suggested to improve the survival of metastatic breast cancer patients.[98] Most recently, however, significant progress has been made in improving the survival outcomes of these patients with the clinical introduction of Trastuzumab. Improvements have occurred among patients who had previously had a prognosis of less than three years.[98] The addition of Trastuzumab to metastatic breast cancer treatment regimens has shown improvements in the progression of breast cancer.[83] In a multicentre study[99] of 114 metastatic HER2-positive patients, there was a 26% overall response rate to Trastuzumab as a monotherapy, without the previous use of chemotherapy. The concurrent combination of anthracyclines with Trastuzumab has been shown to have a significantly greater responsiveness among HER2-positive patients. Stickeler et al 2009 showed that 89% of metastatic breast cancer patients overexpressing HER2 responded to therapy versus 39% of HER2-negative patients.[100]

1.13 Clinical observations of Trastuzumab-induced cardiotoxicity

Despite the therapeutic benefits of Trastuzumab, however, there is an increased incidence of cardiotoxicity, particularly when Trastuzumab is administered following anthracycline-based chemotherapy. Standard parameters of cardiac dysfunction have been defined by the Cardiac Review and Evaluation Committee on subanalysis of phase III clinical trials.[24] Four general criteria were established to be sufficient to conclude a diagnosis of Trastuzumab-related cardiac dysfunction: congestive heart failure (CHF) symptoms; symptoms associated with CHF including an S3 gallop, tachycardia or a combination; and a greater than 5% decline in left ventricular ejection fraction (LVEF) with associated CHF symptoms, or a greater than 10% decline in LVEF without CHF symptoms. The presence of any one of the aforementioned criteria is used to classify Trastuzumab-mediated cardiotoxicity.[24]

In the clinical setting, chemotherapy related dysfunction (CRCD) is classified as Type I or Type II. Type I is characterized by irreversible damage as inflicted by anthracycline induced cardiotoxicity. To some degree, this is considered to result from myocyte apoptosis. On the other hand, Trastuzumab related cardiac dysfunction is generally classified as Type II which is a category of reversible cardiomyopathy, following discontinuation of the drug.[101] Unlike anthracyclines, there is no evidence that Trastuzumab-associated cardiotoxicty is dependant on the cumulative dose administered.[102] Following recovery of cardiac dysfunction *via* treatment with angiotensin-converting enzyme inhibitors and beta blockers,

the reintroduction of Trastuzumab therapy has been shown to be generally well tolerated.[103] Trials such as the HERA trial suggest that upwards of 60% of Trastuzumab-related cases of reduced LVEF, recover to some extent at 6-month follow-up with appropriate heart failure treatment.[104, 105] Outside of clinical trials, which had stringent screening criteria and short follow-up times, in practice, the extent to which Trastuzumab mediated cardiotoxicity is reversible remains undefined.[106] One recent study demonstrated that 22% of adjuvant breast cancer patients recovered from cardiac dysfunction upon the withdrawal of Trastuzumab in a real world population of 152 patients.[107] Real world studies indicate that upwards of 40% showed no recovery despite the withdrawal of Trastuzumab and treatment for heart failure.[107]

A significant predictor of susceptibility to cardiac dysfunction and the potential of reversibility are derived from standard cardiac risk factors including a family history of CAD, history of cardiac disease, smoking and hypertension, and age.[107, 108] These risk factors are also prognostic features of increased susceptibility to Trastuzumab induced cardiotoxicity, and are strongly suggested to be included into screening criteria prior to its use for strategic treatment management.[104, 105, 107]

1.14 Clinical observations of Trastuzumab-induced cardiotoxicity: Adjuvant setting

Anthracyclines, in particular Doxorubicin, have historically been associated with cardiotoxicity. Common risk factors for developing anthracycline mediated cardiac dysfunction include a cumulative dose that exceeds 400 mg/m^2, age greater than 70 years, left chest radiotherapy, and a past history of hypertension.[23, 24]

Out of four major clinical trials using Trastuzumab in the adjuvant setting, following anthracycline based chemotherapy, the incidence of symptomatic CHF was reported to range from 0-4%.[105, 108] Asymptomatic cardiac dysfunction was higher in the clinical trials, reported as an incidence of 5-10%.[107] Further, a review of data from the N9831-NSABP-3[85], HERA,[91, 109, 110] FinHer,[111] BCIRG,[83] and PACS 04 trials suggests that 8-16% of patients required discontinuation of Trastuzumab due to cardiac dysfunction.[108]

Beyond major clinical trial reports, the incidence of Trastuzumab-induced cardiotoxicity may be higher in the real-world clinical adjuvant setting. A retrospective study by Wadhwa et al (2009) found that 24% of patients developed LV systolic dysfunction following the administration of Trastuzumab.[107] Out of a patient population of 152 (mean age 52 ± 10 years), 36 women developed systolic dysfunction as early as 3 months into therapy, with the majority asymptomatic. Delayed enhancement of the myocardium with cardiac MRI revealed that 34/36

patients had myocarditis localized to the subepicardium of the lateral wall of the LV. The main findings from this study indicate that approximately 1 in 4 breast cancer patients undergoing anthracycline based chemotherapy with subsequent Trastuzumab use may develop either symptomatic or asymptomatic LV dysfunction.[107]

1.15 Clinical observations of Trastuzumab-induced cardiotoxicity: Metastatic setting

Unfortunately, the prevalence of cardiotoxicity is higher in the metastatic setting of breast cancer because Trastuzumab is administered concurrently with anthracycline-based chemotherapy. Metastatic breast cancer trials demonstrate that there is a substantially greater incidence of LV cardiac dysfunction, with a CHF prevalence of approximately 22%. This is due to the aggressive treatment approach because metastatic patients have a poor prognosis with a mean life expectancy of 9 to 12 months. Treatment is often palliative with the use of paclitaxel following Trastuzumab therapy, increasing the incidence of cardiac dysfunction to 27%.[75, 83, 91, 100, 112] Despite the high incidence of cardiac complications, the side effects are greatly counter-balanced by the benefits that are otherwise limited for treatment options in the metastatic setting.[108, 113]

1.16 The pathogenesis of Trastuzumab-induced cardiotoxicity

The pathogenesis of Trastuzumab-induced cardiotoxicity includes:

1) Inhibition of NRG-1/ErbB2 signaling within the heart, as required for G protein-coupled receptor signaling, plays a major role in cardiomyocyte cell death. This is based on the following observations: a) inhibition/mutation of HER2/ErbB2 leads to deterioration of ventricular trabeculations leading to dilated cardiomyopathy,[49] b) blockade of HER2/ErbB2 leads to apoptosis through the mitochondrial and ROS-dependant pathways,[50] c) binding of an anti-ErbB2 antibody reduces both ErbB2 activation and down-regulates Bcl-XL, while increasing Bcl-xS expression.[51]

2) The addition of Trastuzumab to anthracycline treatment regimens significantly increases the cardiotoxic risk. An animal HER2/ErbB2 knockout model showed that the sensitivity to anthracyclines was increased in HER2/ErbB2 negative cardiac myocytes.[62] Although the exact mechanism of this is unknown, a number of pathways have been implicated. Along with the agreed notion that these effects are synergistic, this combined effect may be attributed to the anthracycline, induced dilation of T-tubules allowing greater access of Trastuzumab to HER2/ErbB2 within the sarcolemma.[114]

3) Trastuzumab-induced cardiomyopathy is at least partially reversible. The reversibility has been shown in adjuvant clinical trials where discontinuing the drug with or without concurrent treatment for heart failure, has shown recovery of previously diminished left ventricular ejection fraction (LVEF).[88] The mechanism underlying the reversibility itself has scarcely been investigated. It is most conceivable that Trastuzumab inhibition of cardiac HER2/ErbB2 is only temporary. Upon discontinuation of its use, the downstream and adjacent pathways resume their normal function. However, as discussed in this review, the cardiotoxicity likely involves a complex interaction of multiple pathways including apoptotic factors that are together necessary in reversibility of this drug induced cardiomyopathy.

1.17 Prevention of Trastuzumab mediated cardiomyopathy

In the prevention of Trastuzumab mediated cardiotoxicity, two current strategies have been pursued in the clinical setting. The first involves the use of noninvasive cardiac imaging. Current standard preventive strategies use serial multiple-gated acquisition (MUGA) scans and two dimensional echocardiography for baseline evaluation of LVEF followed by serial monitoring every three months in patients receiving adjuvant Trastuzumab. Serial monitoring of LV ejection fraction (LVEF) using noninvasive cardiac imaging is the most important clinical diagnostic tool in early recognition of cardiac dysfunction.[115] MUGA measurements are highly reproducible with a low intraobserver and interobserver variability in comparison to the gold standard, cardiac magnetic resonance

imaging (MRI).[115] Although MUGA is commonly used for cardiac monitoring in this patient population, it is limited by cost, complexity, inaccurate LVEF measurements in patients with underlying arrythmias, and the use of ionizing radiation (equivalent to one or two chest x-rays) over serial examinations(originally published by the American Society of Clinical Oncology. Walker J et al:28(21),2010: 3429-36).[115]

Recently, however, studies have evaluated the use of other imaging modalities, in particular, echocardiography and cardiac magnetic resonance imaging (MRI) for detecting early myocardial damage due to Trastuzumab. A recent study by our group highlighted the potential application of real-time three-dimensional transthoracic echocardiography (RT3D TTE) for monitoring LVEF in the breast cancer setting.[115] The study demonstrated for the first time that RT3D TTE is an accurate and practical method of screening for potential cardiotoxicity among patients with breast cancer receiving adjuvant Trastuzumab treatment.[115] Similar to MUGA, which has a small variability in LVEF, RT3D TTE provided accurate LV volumes and LVEF with high agreement to the gold standard of CMR.[115] Ultimately, the choice of imaging technique for the clinician will be based on local availability. Although MUGA and 2D TTE will likely continue to be the modality of choice for serial assessment of LVEF in this adult patient population, RT3D TTE may be a feasible alternative(originally published by the American Society of Clinical Oncology. Walker J et al:28(21),2001: 3429-36).[115]

1.18 The pathogenesis of Trastuzumab-induced cardiotoxicity: oxidative stress pathways

The second strategy for the prevention of Trastuzumab mediated cardiotoxicity involves the potential use of antioxidant therapy. Although oxidative stress has been well established in the pathogenesis of Anthracycline mediated cardiomyopathy, it has only recently been suggested as a potential mechanism for Trastuzumab-induced cardiotoxicity. Conditional knockout models of the HER2/ErbB2 gene in mice have demonstrated increased levels of ROS, superoxide and proapoptotic protein expression.[49] Similarly, mutation models of HER2/ErbB2 also demonstrate increased levels of ROS, superoxide and proapoptotic protein expression.[114] *In vitro* models also suggest that ROS and apoptosis are increased with the administration of antibodies against HER2/ErbB2.[50, 114] Additionally, an in vivo murine model of acute cardiotoxicity found that cardiomyocyte apoptosis is increased in Trastuzumab treated arms and that the addition of Doxorubicin is synergistic.[40]

Currently, Dexrazoxane is used as a cardioprotective agent against anthracycline induced cardiotoxicity. Dexrazoxane is prescribed in the metastatic setting to disrupt the formation and prevent excessive free-radical production in patients who have exceeded a cumulative Doxorubicin dose of 300 mg/m^2.[116] Although Dexrazoxane has been shown to be highly effective in preventing cardiac dysfunction in the metastatic setting, there is concern that it may reduce the efficacy of chemotherapy.[116] Dextrazoxane may prevent the anthracycline-iron

complex necessary for malignant cytoxicity.[116] There have been conflicting Phase III clinical trial results with one study concluding that the anti-tumor efficacy of Doxorubicin is reduced with Dexrazoxane is used where as other trials suggest that this is not the case.[116]

Likewise, the potent antioxidant Probucol has been shown to attenuate Doxorubicin induced cardiomyopathy in rats.[47] Prophylactic treatment with the potent anti-oxidant Probucol was used in a chronic rat model of Doxorubicin. Doxorubicin was given over a period of 2 weeks for a cumulative dose of 15 mg/kg. Probucol was given 2 weeks prior and concurrently with Doxorubicin for a cumulative dose of 120 mg/kg. There was a reduction both dilated cardiomyopathy and cardiomyocyte apoptosis in those rats receiving Probucol which correlated with an increase in antioxidant reserve and a decrease in markers of oxidative stress and apoptosis compared to placebo groups.[47] This pivotal study suggests that Doxorubicin-induced cardiomyopathy is the result of oxidative stress and cardiomyocyte apoptosis and that prophylactic antioxidant therapy is cardioprotective in this setting.

Although cardioprotective drugs have been evaluated in the setting of anthracycline-induced cardiotoxicity, little is known about attenuating Trastuzumab mediated cardiac dysfunction.

1.19 Probucol

Probucol (2,6-ditert-butyl-4-[2-(3,5-ditert-butyl-4-hydroxyphenyl)sulfanylpropan-2-ylsulfanyl]phenol) is an effective lipid-lowering drug with direct clinical application. Its active anti-oxidant ingredient is bisphenol. Although more effective anti-lipid drugs have been utilized clinically.

Doxorubicin increases plasma triglycerides, total cholesterol, and high- and low-density lipoproteins. Probucol lowers both serum-cholesterol and plasma levels of both high density lipoproteins (HDL) and low density lipoproteins (LDL). The reduction in cholesterol has been shown in human studies among populations of patients with familial hypercholesterolemia. Probucol also lowers cholesterol levels in the setting of Doxorubicin-induced cardiomyopathy. A study by Iliskovic and Singal (1997) compared the efficacy of Probucol to that of Lovastatin, another lipid-lowering drug null of antioxidant properties.[39] In addition to the inability of Lovastatin to lower plasma triglycerides and high-density lipoproteins in the Doxorubicin group, it only partially attenuated Doxorubicin-induced changes in hemodynamics, ascites, and mortality compared to Probucol treated groups.[39] Although Probucol is both a lipid lowering drug and an antioxidant, the cardioprotective effects in the setting of Doxorubicin are due the antioxidant properties and its ability to prevent apoptosis.

In addition to the lipid lowering profile of Probucol, it exerts a protective effect on the function of vascular endothelial cells. The mechanism underlying this involves

the attenuation of endothelial response to acetylcholine, preserving and promoting vasodilation, and scavenging free radicals. This is accomplished via the promotion of endothelial cell growth in addition to endothelium-dependent relaxation. Probucol has been shown to act by promoting nitric oxide (NO) and nitric oxide synthase (NOS) production in endothelial cells throughout the arterial vasculature, ultimately enhancing endothelial function and nitric-oxide mediated relaxation.[117]

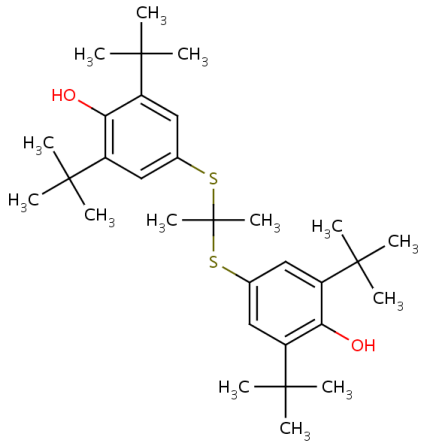

Figure 3. The chemical structure of Probucol, 2,6-ditert-butyl-4-[2-(3,5-ditert-butyl-4-hydroxyphenyl)sulfanylpropan-2-ylsulfanyl]phenol.[54]

1.20 Probucol: endogenous antioxidant reserve

As ROS is a mainstay mechanism for Doxorubicin-induced cardiomyopathy, the use of antioxidants for cardioprotection, including vitamin C, superoxide dismutase, N-acetylcysteine, glutathione catalase, phenetylamines,[118] Coenzyme

Q10 and Probucol, have all proven quite effective in this setting.[19] A dose of 120mg/kg of Probucol has been shown to completely prevent Doxorubicin-induced cardiomyopathy *via* decreasing levels of ROS and decreasing apoptosis. Three studies have replicated these findings. Siveski-Iliskovic et al., 1994a, 1995, Kumar et al. 2001 demonstrated the cardioprotective utility of Probucol when used in combination with anthracyclines.[35, 36, 47, 119] In a chronic model of Doxorubicin chemotherapy, rats were treated to receive a cumulative dose of 120mg/kg. As such, groups that received Probucol in combination with Doxorubicin treatment had increased levels of antioxidants including ROS and significantly less cardiomyopathy compared to groups treated without Probucol.[35, 36, 47, 119]

As discussed above, the pathogenesis of Trastuzumab-associated cardiotoxicity may involve oxidative stress pathways. Thus, the potent antioxidant Probucol may be an effective cardioprotective drug in this application. To our knowledge, no published studies exist that have evaluated the protective effects of an antioxidant drug on attenuating Trastuzumab-induced cardiotoxicity.

Therefore, this project will explore whether Probucol will inhibit both Doxorubicin and Trastuzumab induced cardiotoxicity via reducing oxidative stress in an acute murine model of chemotherapy induced cardiac dysfunction.

1.21 Rationale for study: hypothesis and specific aims

Working hypothesis

With the current increasing number of long-term breast cancer survivors and the tendency to use higher doses of anthracyclines and Trastuzumab throughout the course of treatment, the synergistic cardiotoxic effects are of an increasing concern. The oxidative stress pathway has been implicated in the pathogenesis of adverse left ventricular remodeling due to chemotherapeutic agents.

Prophylactic treatment of mice with Probucol will prevent or reduce cardiac dysfunction, apoptosis and fibrosis in an acute mouse model of Doxorubicin and Trastuzumab mediated cardiomyopathy.

Specific Aims

Two specific aims were pursued, each addressing elements of the working hypothesis:

Aim 1. To determine whether direct antioxidant therapy with Probucol will ***attenuate*** the degree of adverse LV remodeling observed in an acute murine model of anthracycline and Trastuzumab mediated cardiomyopathy, we evaluated *in vivo* LV remodeling and histological evidence of cardiac injury.

Specific Hypothesis:

In an experimental model of cardiac injury due to anthracycline, oxidative stress leads to apoptosis and heart failure. We expect that by ***antagonizing*** cardiac oxidative stress activity with an antioxidant, improved mortality and preserved systolic will be observed in the current animal model. The degree of cardioprotection observed will depend on the ability of the drug to suppress apoptotic levels. Thus, mice receiving a combination of Doxorubicin and Trastuzumab will benefit greatest with Probucol treatment verus mice treated with Doxorubicin or Trastuzumab only.

Aim 2. To elucidate potential mechanisms for the ***cardioprotective*** effects of Probucol in this acute model of chemotherapy induced cardiomyopathy, specific apoptotic markers including BAX and Bcl-XL expression will be evaluated.

Hypothesis:

In mice receiving direct antioxidant therapy, apoptosis is suppressed leading to downregulation of the apoptotic cascade. Specifically, proapoptotic Bax, levels will be decreased, and antiapoptotic BcL-XL levels will be increased. Probucol will suppress the greatest degree of apoptosis in mice receiving the combination of Doxorubicin and Trastuzumab.

Chapter 2 Methodology

All animal procedures were conducted in accordance with guidelines published by the Canadian Council on Animal Care. All procedures, including drug administration and longitudinal echocardiography studies, were approved by the Animal Protocol Review Committee at the University of Manitoba.

2.1 Animal model

A total of 8 groups of 7-8 week old mice (C57Bl/6) were studied for 4 weeks. Groups received one of the following treatment regimens: (**1)** 0.9% saline control; (**2)** Probucol; (**3)** Doxorubicin (D); (**4)** Trastuzumab (T); (**5)** Doxorubicin+Trastuzumab (D+T); (**6)** Probucol+Trastuzumab (P+T); (**7)** Probucol+Doxorubicin (P+D); **(8)** Probucol+Doxorubicin+Trastuzumab (P+D+T) as shown in Figure 4.

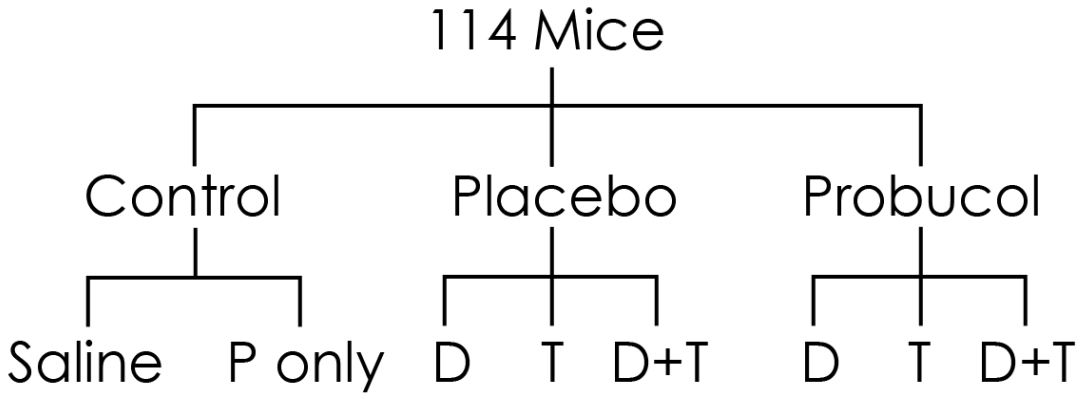

Figure 4. Algorithm of treatment regimens. P, Probucol; D, Doxorubicin; T, Trastuzumab. Saline (N=5), Probucol (N=5), T only (N=8), D only (N=25), D+T (N=33), P+T (N=11), P+D (N=11), P+D+T (N=16).

Following one week of quarantine, each group was randomized to receive either control, placebo or prophylactic treatment with Probucol (15 mg/kg i.p). Probucol injections were given on alternative days, Monday, Wednesday, and Friday for two weeks prior to the experimental arm and one injection during the experimental arm for a cumulative dose of 120 mg/kg (Figure 5).

Acute treatment injections were administered i.p. at day 0 as single injections with either D (20 mg/kg) or T (10 mg/kg) or the combination of D+T.

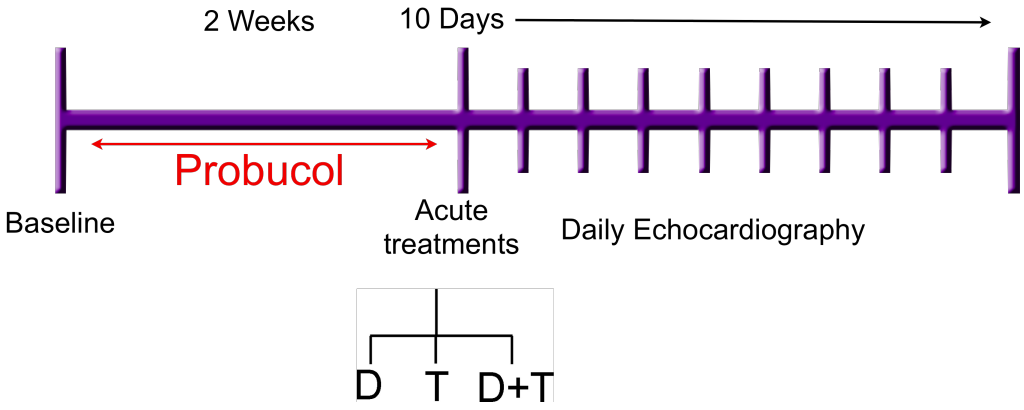

Figure 5. Timeline of mice receiving either control, placebo, or Probucol. Mice received prophylactic Probucol (120 mg/kg) for 2 weeks prior to acute treatment with either Doxorubicin (D), Trastuzumab (T) or Doxorobucin and Trastuzumab (D+T). After acute treatment, mice were followed for 10 days with daily echocardiograms. At day 10, all mice were euthanized and organs were preserved for histological and biochemical analysis.

Serial echocardiography and weight measurements were performed daily for 10 days. On day 10 of the experimental arm, all groups of mice were euthanized and heart and livers were preserved for further histological and biochemical analysis.

2.2 Murine echocardiography

In vivo assessment of cardiac function was performed using murine echocardiography with 2-dimensional and tissue Doppler imaging (TDI). All mice were examined at baseline and followed daily thereafter for 10 days (Figure 5). Murine echocardiography was performed using a 13-Mhz probe (Vivid 7, GE Medical Systems, Milwaukee, Wi, US) and a 30 MHz MicroScan transducer (Vevo 2100, Visualsonics, Toronto, Canada). Hearts were imaged in the 2D parasternal long and short axis views. Left ventricular ejection fraction (LVEF) was calculated by tracing the end diastolic and end systolic volumes in the parasternal long axis view (Figure 6, Eqn 1).

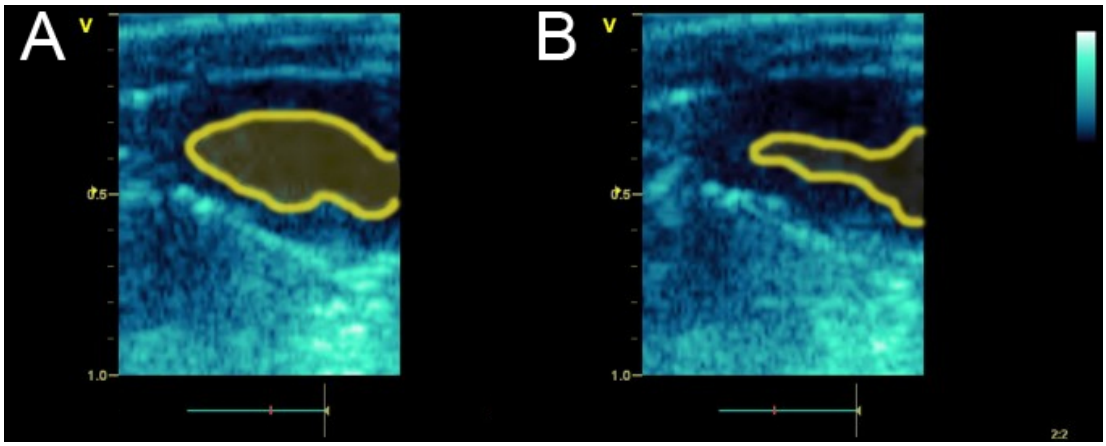

Figure 6. Parasternal long axis view on 2D transthoracic echocardiography demonstrating LV end diastolic (panel A) and end systolic volumes (panel B), respectively.

In the 2D parasternal short axis view, M-mode was used to obtain the following measurements: LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVEDS), posterior wall thickness (PWT), and fractional shortening (FS).

Tissue Doppler imaging (TDI) was acquired on a parasternal short axis view at the level of the papillary muscles, at a rate of 483 frames per second. Peak systolic endocardial velocity (V_{ENDO}) and radial strain rate (SR) were obtained from a region of interest of 0.2 mm x 0.2 mm on the posterior wall.[40, 53]

Eqn 1:

$$\text{Ejection fraction} = \frac{\text{end diastolic volume (cc)} - \text{endsystolic volume (cc)}}{\text{end diastolic volume (cc)}} \times 100\%$$

2.3 Histological studies

Following 10 days post-treatment, all mice were euthanized and hearts were removed for histological and biochemical analysis. A portion of the liver was taken for wet-to-dry ratio. Hearts were rinsed in phosphate buffered solution (PBS) and sectioned into halves. One half of the heart was taken for biochemical analysis and the other half was placed in a labeled container filled with formalin fixative. These heart tissues were processed in the pathology laboratory of the St. Boniface General Hospital for paraffin sectioning. Fixed 5-micron thin sections were stained with Masson's trichrome to assess cell damage, vacuolization and myofibril degeneration.[119]

Wet-to-dry ratios of pre-weighed liver were measured for all mice. Portions of the liver were weighed and dried at 85ºC for 72 hours. Dry weights were measured and wet-to-dry ratios were calculated.

2.4 Protein extraction and immunoblotting

Quantification of Bax and Bcl-XL were performed as previously described.[120] Frozen half hearts from three mice were pooled and powdered in liquid nitrogen and homogenized in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Sigma-Aldrich Corporation, St Louis, MO). The lysates were centrifuged at 14,000 rpm for 10 minutes. The upper layer containing protein fraction was sonicated and stored at -75ºC. Protein concentration was measured using the BioRad protein assay. The protein samples were thawed and subjected to 1-dimensional 12% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis in a discontinuous system as previously described.[120] Equal loading of protein (30 mg/lane) was confirmed by Coomassie blue staining as well as by the use of antiactin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as an internal control. Separated proteins were transferred onto 0.45-mm nitrocellulose membrane and incubated overnight with Bax and Bcl-XL polyclonal antibodies (Cell Signaling Technology Inc, Beverly, MA). Primary antibodies were detected using a goat antirabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Inc, Hercules, CA) using the BM Chemiluminescence kit (POD substrate; Roche Diagnostics, Laval, Canada). The protein bands were visualized with a Fluor-S-MultiImager MAX system (Bio-Rad Laboratories, Inc) and quantified using image analysis software (Quantity One; Bio-Rad Laboratories, Inc). The above procedures were repeated three times with three hearts each.

2.5 Statistical analysis

Echocardiographic data are expressed as mean±SEM. All statistical analysis were evaluated using SPSS 15.0 and Graphpad Prism 5. For comparison of the echocardiographic parameters over time, results were analyzed using analysis of variance for repeated measurements. Post hoc comparisons, when necessary, were performed using one-way anova and post-hock Dunnett's tests. A P-value <0.05 was considered statistically significant.

Chapter 3 Results

3.1 Murine echocardiography

At baseline, the LV dimensions and systolic function, as determined by both conventional parameters (FS and EF) and TDI indices (Vendo and SR) were similar in all mice. Heart rates were within normal limits at baseline. There was no evidence of left ventricular hypertrophy comparing PWT at baseline and day 10 of follow-up (Table 1).

Echo variable	Group	Baseline values	Day 10 values	p-Value
HR (beats/min)				
	Saline	734±15	728±11	0.82
	Probucol	727±8	715±10	0.76
	T only	744±11	754±9	0.58
	D only	715±12	725±14	0.45
	D+T	731±8	725±10	0.67
	P+T	719±10	734±8	0.72
	P+D	724±8	715±7	0.41
	P+D+T	735±8	715±10	0.31
PWT (mm)				
	Saline	0.81±0.02	0.84±0.03	0.82
	Probucol	0.83±0.02	0.84±0.02	0.83
	T only	0.8±0.04	0.81±0.01	0.85
	D only	0.82±0.02	0.80±0.02	0.78
	D+T	0.81±0.02	0.83±0.03	0.84
	P+T	0.83±0.02	0.82±0.02	0.88
	P+D	0.81±0.03	0.83±0.04	0.78
	P+D+T	0.82±0.03	0.84±0.04	0.76
LVEDD (mm)				
	Saline	3.2±0.2	3.2±0.1	0.84
	Probucol	3.1±0.1	3.2±0.2	0.78
	T only	3.2±0.2	3.2±0.1	0.87
	D only	3.2±0.1	3.8±0.2**	<0.05
	D+T	3.1±0.1	4.1±0.2**	<0.05
	P+T	3.2±0.1	3.2±0.1	1.00
	P+D	3.2±0.1	3.5±0.1**	<0.05
	P+D+T	3.1±0.1	3.6±0.1**	<0.05
FS (%)				
	Saline	51±3	53±2	0.67
	Probucol	50±2	51±3	0.72
	T only	53±2	52±3	0.84
	D only	51±2	42±2**	<0.05
	D+T	52±3	35±3**	<0.05
	P+T	51±2	50±1	0.81
	P+D	52±2	47±2**	<0.05
	P+D+T	51±1	44±2**	<0.05

Table 1. Echocardiographic parameters at baseline and day 10 in C57Bl/6 mice receiving one of the drug regimens. T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab; HR, heart rate; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; and, FS, fractional

shortening. Values are mean±SEM. Baseline, 5 Saline, 5 Probucol, 5 T only, 20 D only, 26 D+T, 11 P+D, 16 P+D+T; Day 10, 5 Probucol, 5 T only, 7 D only, 3 D+T, 10 P+D, 10 P+D+T.

The LVEDD was within normal limits in all groups at baseline (Table 1). In mice receiving D alone, the LVEDD increased significantly from 3.2 mm at baseline to 3.8 mm by day 10 ($p<0.05$). In mice receiving D+T, the LVEDD increased significantly from 3.1 mm at baseline to 4.1 mm by day 10 ($p<0.05$). The administration of Probucol however attenuated the increase in LVEDD to only 3.5 mm at day 10 in the P+D group and 3.6 mm at day 10 in the P+D+T (Table 1. $P<0.05$).

Fractional shortening (FS) was within normal limits in all groups at baseline (Table 1). In mice receiving D alone, the FS decreased from 51% at baseline to 42% at day 10 ($p<0.05$). In mice receiving D+T, the FS decreased from 52% at baseline to 35% at day 10 ($p<0.05$). The administration of Probucol however attenuated the decrease in FS to only 47% at day 10 in the P+D group and 44% at day 10 in the P+D+T (Table 1. $P<0.05$).

Left ventricular ejection fraction (EF) was within normal limits in all groups at baseline (Figure 7). In mice receiving D alone, the EF decreased from 83% at baseline to 66% at day 4 ($p<0.05$). In mice receiving D+T, the EF decreased from 84% at baseline to 58% at day 4 ($p<0.05$). The administration of Probucol

however attenuated the decrease in LVEF until day 7 where it decreased to 77% and did not significantly change in the P+D+T group by day 10 (Figure 7).

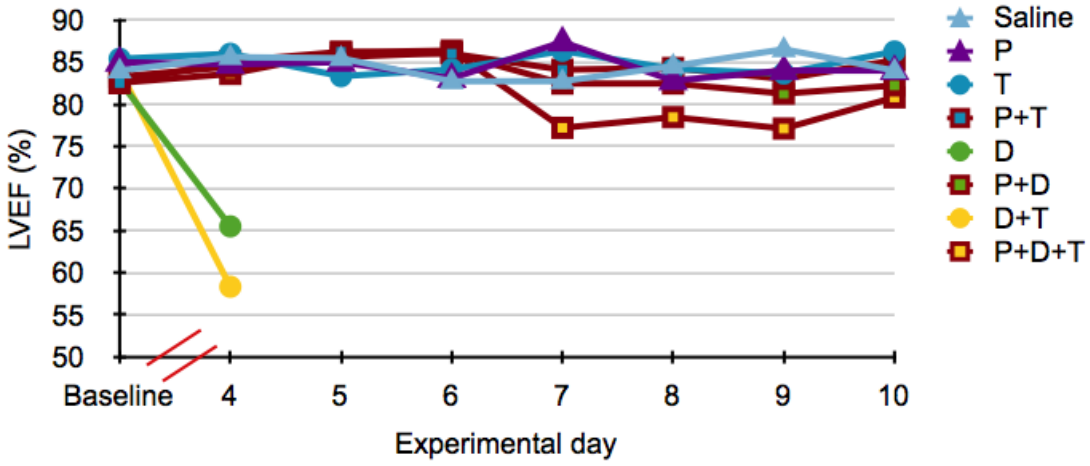

Figure 7. The LVEF of groups of C57Bl/6 mice. P, Probucol; T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab. At day 5 mice remaining were 5 Sal, 5 P only, 11 D only, 5 T only, 7 D+T, 11 P+D, 10 P+T, 16 P+D+T. At day 10 mice remaining were 5 Saline, 5 P only, 7 D only, 5 T only, 3 D+T only, 10 P+D, 10 P+T, 10 P+D+T. Error bars represent SEM on days where there was a mortality. $P<0.01$ between groups.

Similar to the FS and EF, TDI indices of Vendo and SR revealed LV systolic dysfunction by day 10 (Table 2). In mice receiving D alone, Vendo and SR decreased from 3.2 cm/s and 23 s^{-1} at baseline, respectively, to 1.7 cm/s and 13 s^{-1} at day 10, respectively ($p<0.05$). In mice receiving D+T, Vendo and SR decreased from 3.3 cm/s and 24 s^{-1} at baseline, respectively, to 1.4 cm/s and 10 s^{-1} at day 10, respectively ($p<0.05$). The administration of Probucol however attenuated the decrease in Vendo and SR in the P+D group to only 2.1 cm/s and 17 s^{-1}, respectively. Similarly, the administration of Probucol attenuated the decrease in Vendo and SR in the P+D+T group to only 1.8 cm/s and 15 s^{-1}, respectively.

Echo variable	Group	Baseline values	Day 10 values	p-Value
Vendo (cm/s)				
	Saline	3.2±0.1	3.2±0.2	0.92
	Probucol	3.2±0.1	3.2±0.2	0.91
	T only	3.3±0.2	3.3±0.1	0.88
	D only	3.2±0.1	1.7±0.1**	<0.05
	D+T	3.3±0.1	1.4±0.1	<0.05
	P+T	3.2±0.1	3.2±0.2	0.88
	P+D	3.3±0.1	2.1±0.1**	<0.05
	P+D+T	3.2±0.1	1.8±0.1**	<0.05
SR (S^{-1})				
	Saline	22±2	22±1	0.92
	Probucol	23±1	22±2	0.81
	T only	23±1	23±2	0.90
	D only	23±2	13±2**	<0.05
	D+T	24±1	10±1**	<0.05
	P+T	23±1	22±1	0.81
	P+D	22±1	17±2**	<0.05
	P+D+T	23±1	15±1**	<0.05

Table 2. Echocardiographic parameters at baseline and day 10 in C57Bl/6 mice receiving one of the drug regimens. T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab; Vendo, endocardial velocity; SR, strain rate. Values are mean±SEM. Baseline, 5 Saline, 5 Probucol, 5 T only, 20 D only, 26 D+T, 11 P+D, 16 P+D+T; Day 10, 5 Probucol, 5 T only, 7 D only, 3 D+T, 10 P+D, 10 P+D+T.

3.2 Survival probability and mortality

The survival probability of mice receiving one of the treatment regimens is shown in Figure 8. There was no mortality observed in the control mice. Mice receiving D alone had a 50% survival rate at day 5. The addition of Probucol to D (P+D), significantly preserved the *survival probability* to 91% at day 10 (Figure 8). Mice treated with the combination of D+T demonstrated >80% mortality at day 5. Mice treated with P+D+T demonstrated preserved survival probability to 63% at day 10. The addition of Probucol to groups shifted the survival curve to the right and preserved the survival in mice receiving either D alone or D+T (Figure 8).

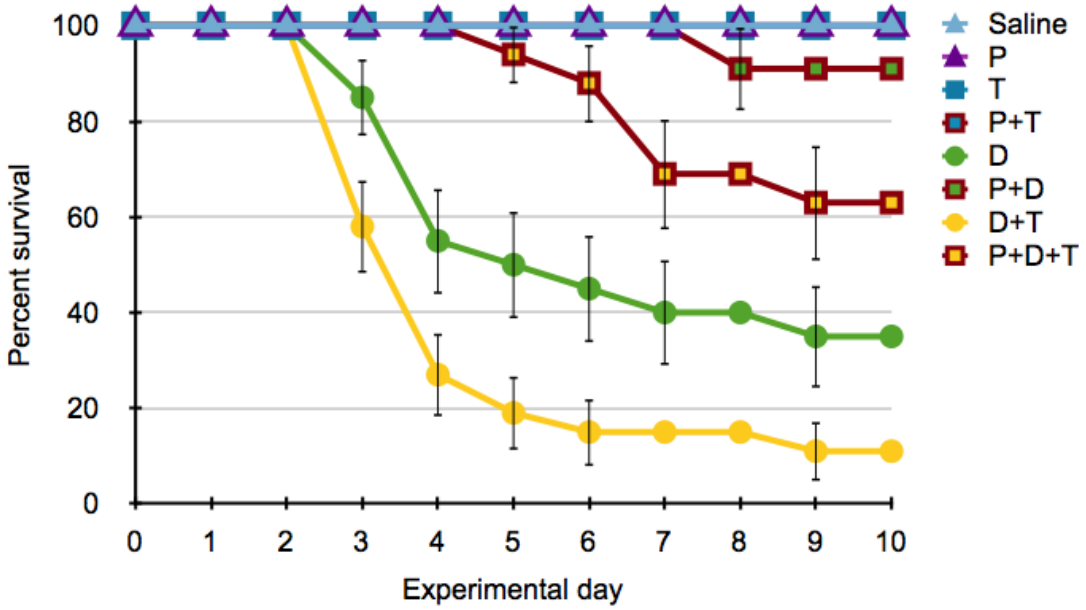

Figure 8. The survival probability of groups of C57Bl/6 mice. P, Probucol; T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab. At day 5 mice remaining were 5 Sal, 5 P only, 11 D only, 5 T only, 7 D+T, 11 P+D, 10 P+T, 16 P+D+T. At day 10 mice remaining were 5 Saline, 5 P only, 7 D only, 5 T only, 3 D+T only, 10 P+D, 10 P+T, 10 P+D+T. Error bars represent SEM on days where there was a mortality. $P<0.01$ between groups.

3.3 Tissue weights

The average ratios of wet to dry weight for the liver tissue were not significantly different between treatment groups at day 10 of follow-up (Figure 9).

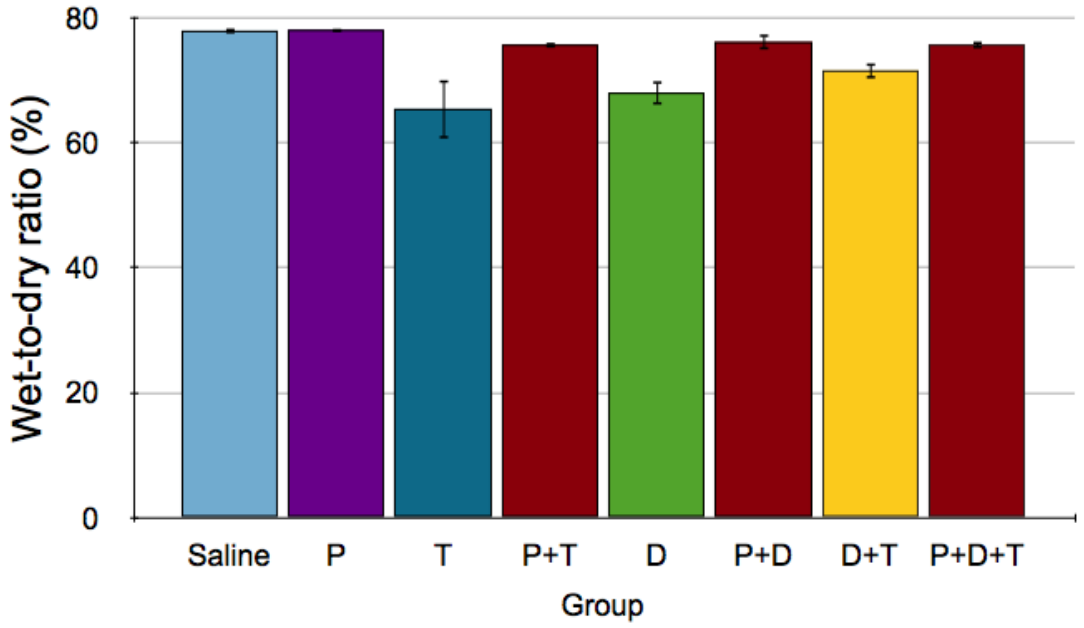

Figure 9. Liver tissue wet-to-dry ratios of groups of C57Bl/6 mice. Probucol; T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab. T, Trastuzumab. 5 Saline, 5 Probucol, 5 T only, 20 D only, 25 D+T, 11 P+T, 11 P+D, 16 P+D+T.

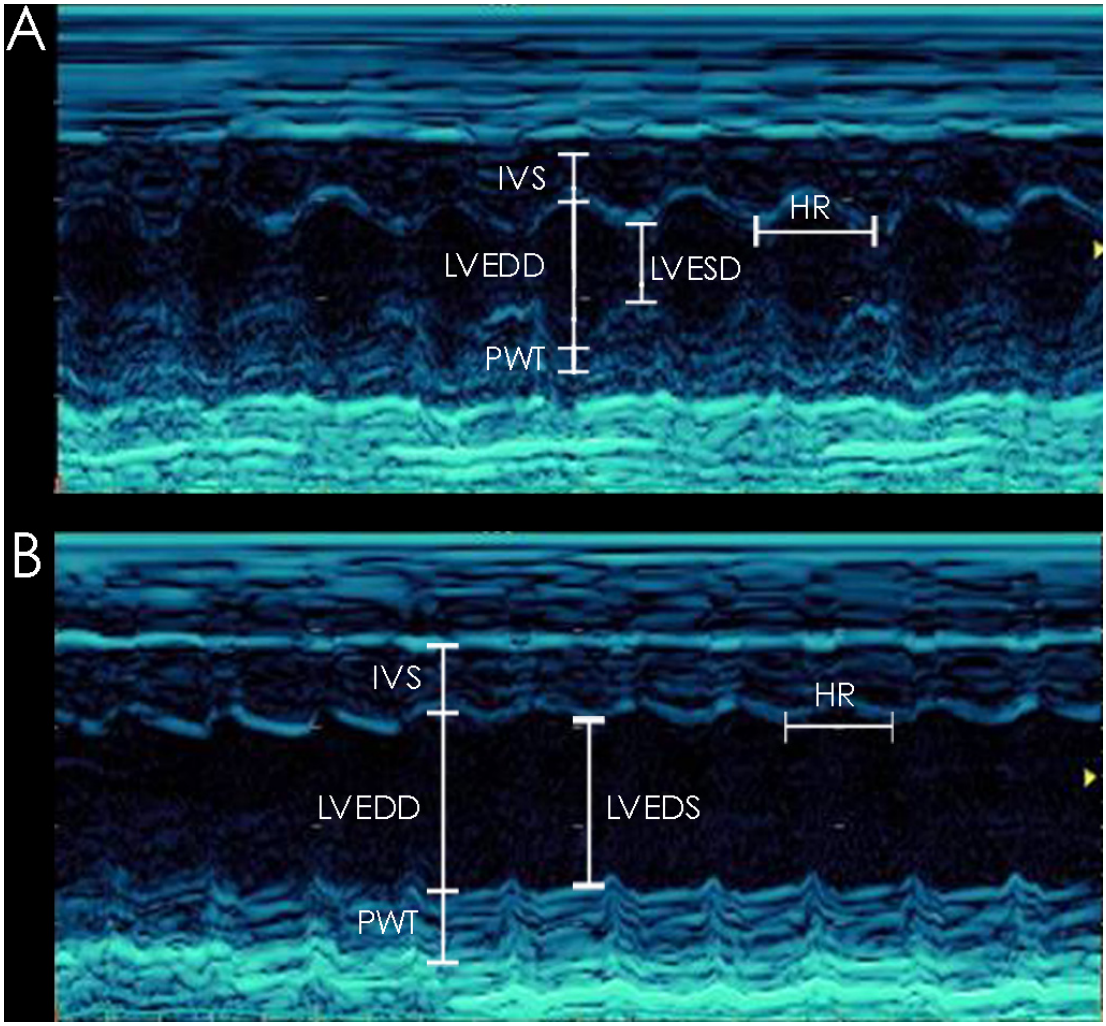

Figure 10. Representative M-mode echocardiograms of D+T mice at: a) baseline (left) and B) 5 days post treatment (right). At baseline, the LV cavity dimensions were within normal limits. At 5 days post treatment, the LV cavity is dilated with LV systolic dysfunction. Interventricular septum (IVS); Left ventricular end diastolic diameter (LVEDD); Posterior wall thickness (PWT); Left ventricular end systolic diameter (LVEDS).

3.4 Histology

By day 5 of the experiment, myofibrillar degeneration and vacuolization was present in mice receiving D and D+T (Figure 11 C and D). By day 10, mice receiving prophylactic probucol, P+T, P+D, and P+D+T had minimal myofibrillar degeneration and vacuolization (Figures 11 F, G, and H).

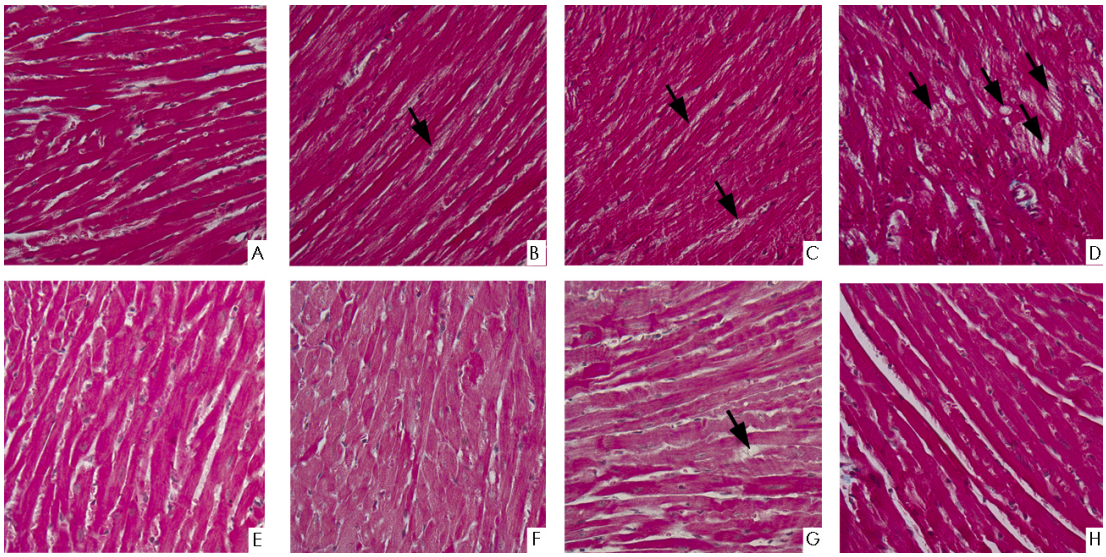

Figure 11. Heart sections from placebo groups: (A) Saline control, (B) T only, (C) D only, (D) D+T; and, probucol groups (E) Probucol control, (F) P+T, (G) P+D, and (F) P+D+T. Arrows indicate cardiomyocytes showing drug-induced damage which was greatest in the D+T group.

3.5 Western-blotting

At day 10 of the experiment, protein expression of Bax and Bcl-XL were evaluated in all treatment groups. In mice receiving T, D, and D+T in combination, the expression of Bax was 7.5, 14.1 and 12.1 OD respectively (Figure 12). The administration of Probucol to each of these arms however, significantly decreased Bax expression to 1.8, 4.8, and 8.2 OD respectively (Figure 12).

Similarly, in mice receiving T, D, and D+T in combination, the expression of Bcl-XL was 2.2, 1.9 and 0.6 OD respectively. The administration of Probucol to each of these arms however, significantly increased Bcl-XL expression to 2.7, 2.1, and 2.4 OD respectively (Figure 13).

Finally, as shown in figure 13, in mice receiving T, D, and D+T in combination, the ratio of Bax/Bcl-XL was 3.5, 7.5 and 22 OD respectively. There was a 5-fold increase in the ratio of Bax/Bcl/XL in mice receiving D+T. The administration of Probucol to each of these arms however, significantly decreased the ratios to 1, 2.5 and 3.7 OD, respectively (Figure 14).

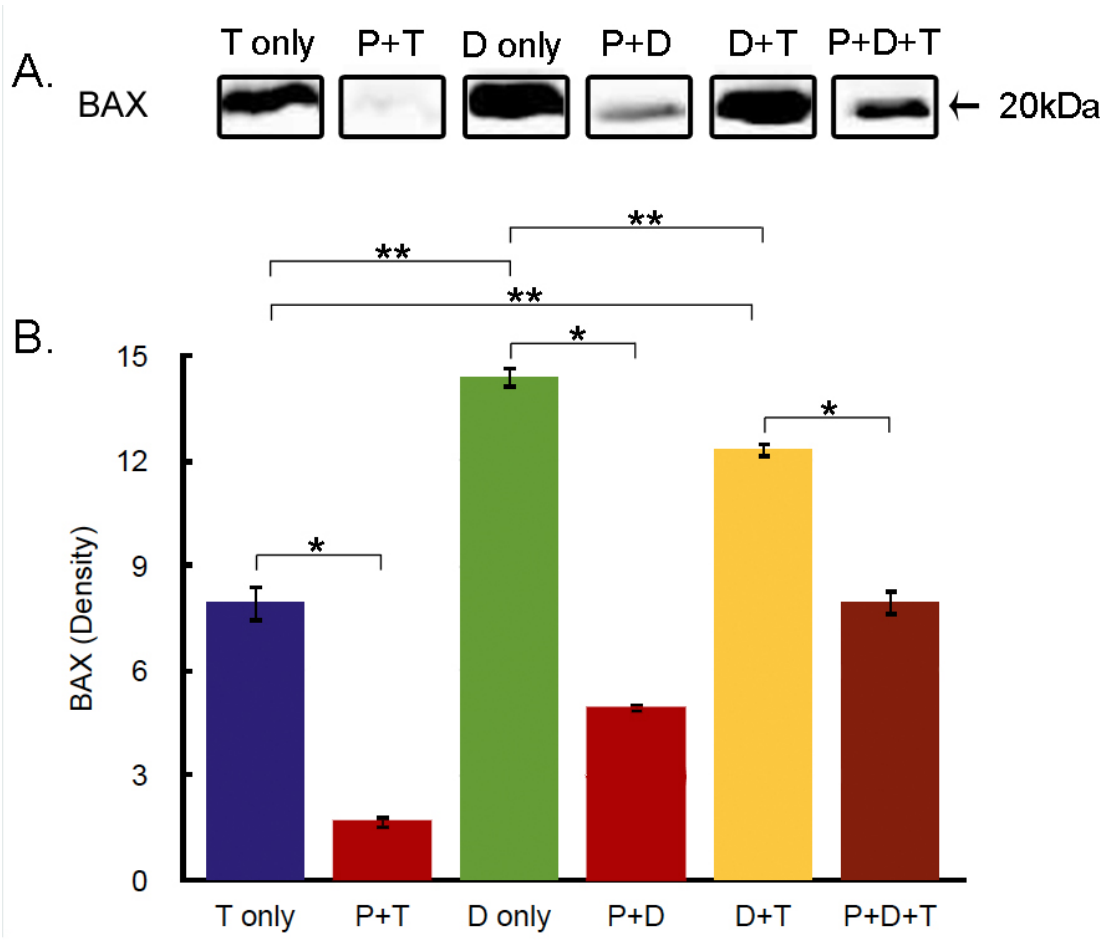

Figure 12. The average BAX protein expression at day 10 in O.D units. P, Probucol; T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab. Day 10 hearts analyzed: 6 T only, 6 P +T, 6 D, 6 P +D, 6 D+T, 6 P+D+T. Error bars represent SEM. *, $P<0.05$ probucol treated groups in comparison to respective placebo group, ** $P<0.05$ between placebo groups.

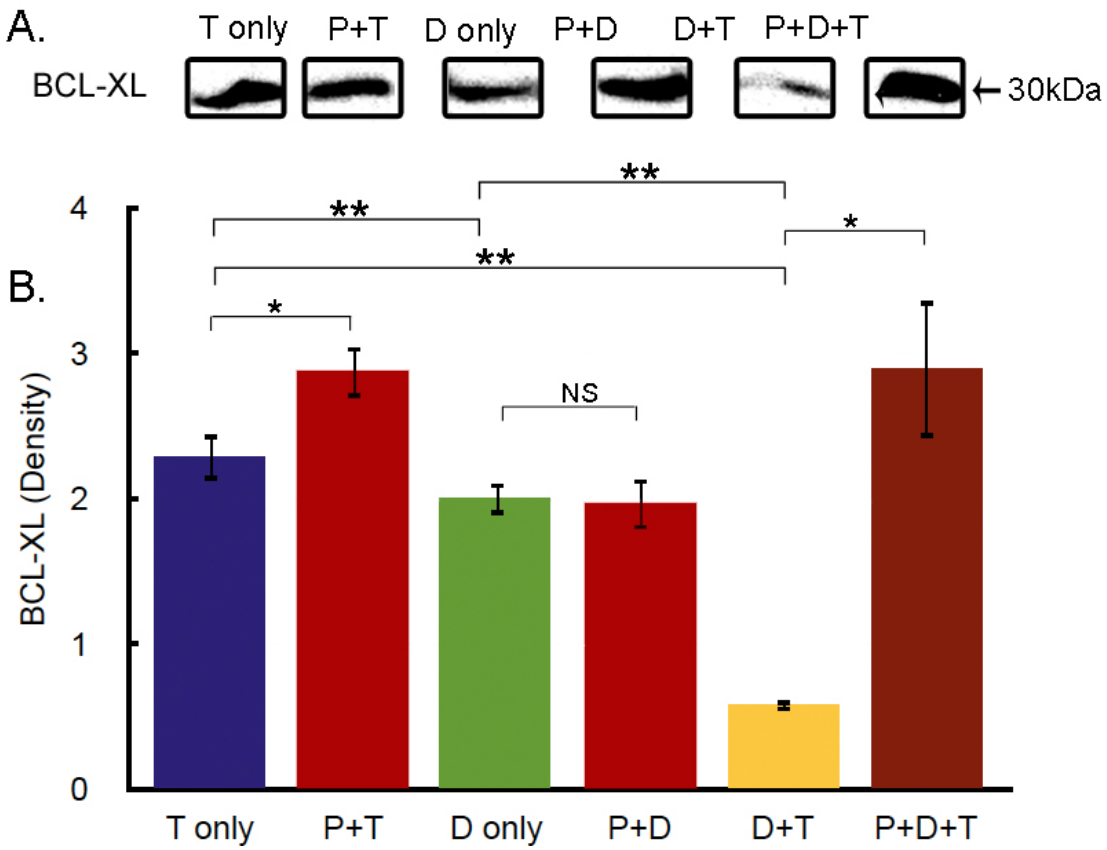

Figure 13. The average BCL protein expression at day 10 in O.D units. P, Probucol; T only, Trastuzumab; D only, Doxorubicin; D+T, Doxorubicin+Trastuzumab; P+D, Probucol+Doxorubicin; P+D+T, Probucol+Doxorubicin+Trastuzumab. Day 10 hearts analyzed: 6 T only, 6 P +T, 6 D, 6 P +D, 6 D+T, 6 P+D+T. Error bars represent SEM. *, $P<0.05$ probucol treated groups in comparison to respective placebo group, ** $P<0.05$ between placebo groups.

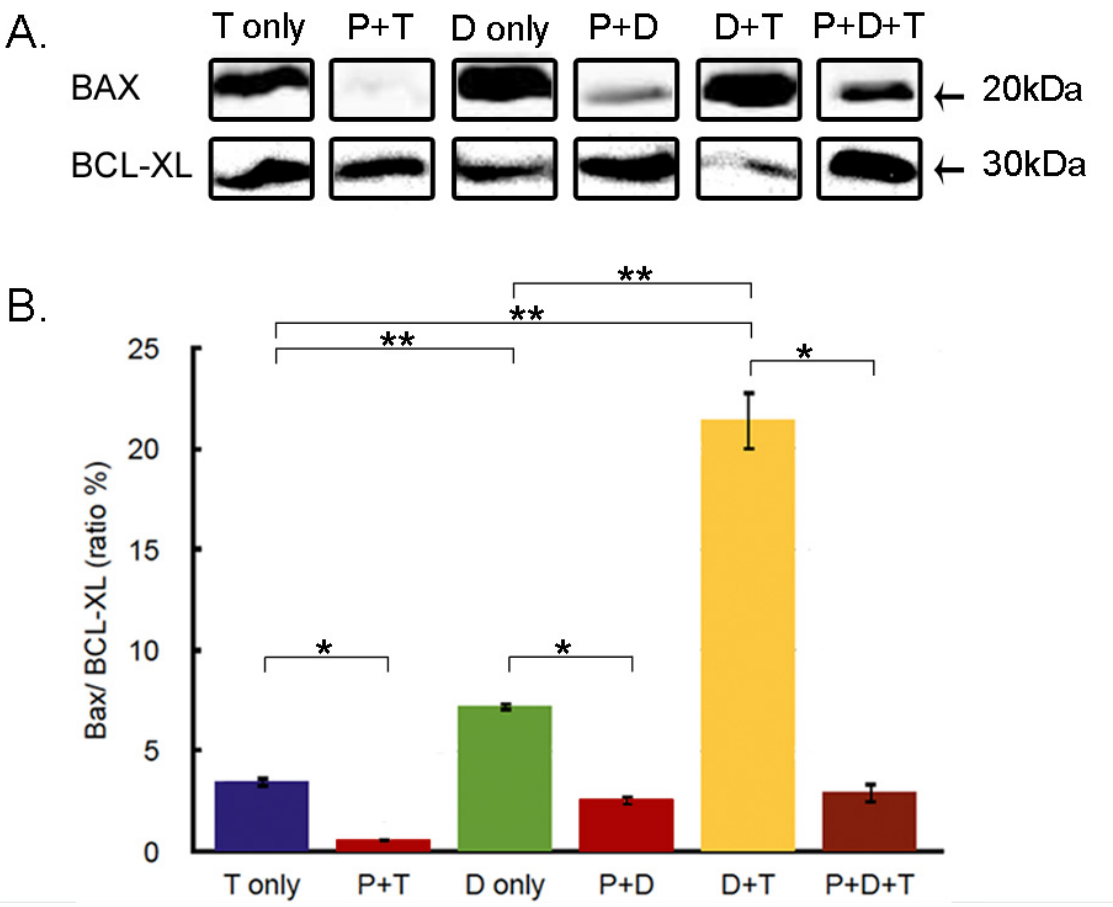

Figure 14. The effect of treatment of C57/Bl/6 mice with different drugs on Bax and BCL/XL apoptotic and antiapoptotic proteins in the hearts. Day 10 hearts analyzed: 6 T only, 6 P +T, 6 D, 6 P +D, 6 D+T, 6 P+D+T. *, P<0.05 probucol treated groups in comparison to respective placebo group, ** P<0.05 between placebo groups.

Chapter 4 Discussion

4.1 Summary of current findings

The present study demonstrates that prophylactic therapy with the antioxidant Probucol significantly reduces the combined cardiotoxic side effects of Doxorubicin and Trastuzumab therapy. Before acute treatment with one of the chemotherapeutic regimens, LV dimensions and contractile function of all groups did not differ. Mice treated with prophylactic Probucol demonstrated minimal cardiotoxicity and improved survival compared with those treated with Doxorubicin and Trastuzumab. There was histologic evidence of decreased Doxorubicin and Trastuzumab mediated cardiac damage in the Probucol treated groups. Finally, the degree of apoptotic promoting proteins to antiapoptotic proteins was significantly attenuated in mice receiving prophylactic therapy with Probucol. Thus, the prophylactic administration of Probucol therapy in the acute setting effectively attenuated the degree of increased mortality, systolic dysfunction and increased cardiac apoptosis observed in mice receiving the combination of Doxorubicin and Trastuzumab.

4.2 Left ventricle cavity dimensions and systolic function

An acute *in vivo* murine model of Doxorubicin-induced cardiomyopathy was previously established by Neilan and colleagues.[52] In this study, wild type mice receiving Doxorubicin (20 mg/kg) demonstrated echocardiographic evidence of

severe LV cavity dilation by day 5.[52] As compared to baseline, both FS and EF decreased significantly in wild-type mice receiving Doxorubicin. In the same study, a chronic protocol, involving the administration of a lower dose of Doxorubicin (4mg/kg) weekly for 5 weeks was evaluated.[52] At week 16, the results were consistent with their acute model where wild-type mice demonstrated echocardiographic evidence of a dilated cardiomyopathy.[52] Thus, both acute and chronic models of Doxorubicin-induced cardiomyopathy demonstrate LV dilation and a reduction in FS and EF.

In 2009, Jassal et al. evaluated an acute in vivo murine model of anthracycline and Trastuzumab mediated cardiomyopathy.[40] In total, 40 mice received one of the following drug regimens: (i) Control; (ii) Doxorubicin; (iii) Trastuzumab; and (iv) D+T. Progressive LV dilatation and LV systolic dysfunction was observed by day 4 of treatment with D+T, compared with preserved LVEF in the remaining groups.[38] Our current findings are consistent with the previously mentioned studies. Serial echocardiographic analyses of the mice receiving D alone or the combination of D+T revealed progressive LV dilatation and reduced FS and EF.[40, 52] Specifically, in mice receiving D alone and D+T, the LVEDD increased 1.2 fold and 1.3 fold respectively. Prophylactic treatment with Probucol however significantly attenuated the degree of LV dilatation by 10% in the combined D+T group. Similarily, Probucol attenuated the decrease in FS and EF in the D only and D+T groups. These novel findings suggest that LV systolic dysfunction from the synergistic combination of Doxorubicin and Trastuzumab is partially

attenuated by the prophylactic administration of Probucol.

4.3 Early detection with tissue Doppler imaging

Although current clinical strategies utilize the serial evaluation of LV systolic function for the detection of Trastuzumab induced cardiotoxicity, recent studies have found more sensitive, early markers of Trastuzumab-induced cardiac dysfunction using noninvasive cardiac imaging. Consistent with the current study's findings, tissue Doppler imaging (TDI), using echocardiography, is a sensitive, noninvasive echocardiographic technique that allows for measurements of velocity at any point along the ventricular wall during the cardiac cycle.[40, 52, 53, 121] TDI allows for the measurement of maximal systolic endocardial velocity (Vendo) and strain rate (SR). When compared with conventional measures of LVEF, TDI-derived parameters are less influenced by loading conditions, such as the change in intravascular volume that occurs with chemotherapy and Trastuzumab therapy.[40] Thus, TDI is a more feasible imaging modality that might provide improved sensitivity in detecting early subclinical LV dysfunction.

In the clinical setting of anthracycline-induced cardiomyopathy, the utility of TDI has been recently studied.[31, 122] Two studies suggested that TDI provides a more accurate evaluation of anthracycline-induced cardiotoxicity earlier than radionucleotide LVEF estimations with multi-gated acquisition scans (MUGA).[31, 122] Three clinical studies have evaluated the utility of myocardial deformation in the

preclinical detection of Trastuzumab mediated cardiac dysfunction. Nagy et al. 2006 evaluated the use of TDI in 40 previously healthy women undergoing treatment with antracyclines who had no previous history of cardiac dysfunction.[122] TDI was sensitive to early changes in wall motion and E/E' prior to changes in left ventricular ejection fraction (LVEF).[122] Similarly, Ho et al. evaluated 51 breast cancer patients receiving anthracyclines without Trastuzumab and 19 who received Doxorubicin followed by adjuvant Trastuzumab therapy.[31] This study demonstrated that subclinical detection of anthracycline-induced cardiotoxicity was present 6 years following treatment in asymptomatic patients.[31]

In the early detection of Trastuzumab-induced cardiomyopathy, tissue Doppler imaging (TDI) is a sensitive noninvasive echocardiographic technique. In an acute in vivo murine model of Trastuzumab-induced cardiomyopathy, Jassal et al. (2009) evaluated the sensitivity of TDI to detect early LV dysfunction prior to alterations in conventional echocardiographic parameters.[40] TDI results were abnormal in mice receiving either D alone or D+T as early as 24 hours after treatment.[38] After 48 hours, both Vendo and SR were significantly decreased and were predictive of ensuing LV systolic dysfunction and increased mortality.[40] Conventional measures of LV systolic function using serial measurements of LVEF were not sensitive to changes until day 4 of this study. Hence, TDI was able to detect subclinical evidence of LV systolic dysfunction prior to alterations in conventional LVEF measures.

The findings of the current study confirm that TDI is a sensitive measure of chemotherapy induced cardiotoxicity. Although LV systolic parameters did not show significant changes in function until day 4, TDI parameters of Vendo and SR were sensitive to myocardial changes earlier than LVEF. To further, mice receiving P+D+T demonstrated preserved Vendo and SR as compared to D+T alone. This further supports the utility of TDI and strain imaging to detect preclinical changes in LV ejection fraction in Anthracycline and Trastuzumab mediated cardiomyopathy.

4.4 Survival

Similarly, acute murine models of chemotherapy have demonstrated increased mortality associated with Doxorubicin. Nielan et al. (2007) administered a single dose of Doxorubicin (20mg/kg) to C57BL/6 mice (N=40), the mortality rate was 43% by day 8.[52] Further, Jassal et al. (2008) studied mice receiving Doxorubicin (20mg/kg) or the combination of Doxorubicin (20mg/kg) and Trastuzumab (10mg/kg).[40] The authors demonstrated that the mortality rate among the Doxorubicin arm was comparable to Neilan et al. (2007)[52] where it was 80% by day 5.[40]

Antagonism of the HER2 receptor is associated with a dilated cardiomyopathy. Crone *et al.* 2002 demonstrated the development of dilated cardiomyopathy in a conditional ventricular-restricted deletion of HER2/erbB2 in embryonic mice.[49] HER2/ErbB2 deficient mice with a total gene knockout model were found to have impaired ventricular trabeculation with decreased survival.[49] A similar conditional knockout (CKO) study by Ozcelik et al. 2002 reported reduced survival rates in mice with a conditional knockout of the gene encoding the HER2/ErbB2 receptor (N=22).[114] The majority of mice demonstrated the onset of dilated cardiomyopathy by 2 months of age. These authors demonstrated that suppression of HER2/ErbB2 signaling through conditional mutation, impairs cardiac function leading to a dilated cardiomyopathy with a high rate of sudden cardiac death.[114]

As compared to the aformentioned studies, our current study demonstrated similar mortality results among mice receiving T, D and D+T.[40, 49, 52, 114] Mice treated acutely with Doxorubicin had decreased survival compared to controls. Mice receiving D alone had a 50% survival rate at day 5. The combination of D+T, however, demonstrated the worst survival probability of all groups with >80% mortality at day 5.

The prevention of increased mortality risk associated with Doxorubicin-induced cardiotoxicity by the use of antioxidant therapy has been well established in

rodent models. Rodent models of Doxorubicin-induced cardiomyopathy, including Kumar et al 2001, compared the mortality of rats receiving placebo and Doxorubicin (15mg/kg) over two weeks to a prophylactic Probucol (120mg/kg) and Doxorubicin (15mg/kg) group.[35] Kumar et al. 2001 demonstrated that placebo treated rats receiving Doxorubicin arm had a 40% mortality after 21 days.[35] The combination of Probucol with Doxorubicin substantially prevented mortality rate. Similarly, in the study by Siveski-Iliskovic et al. 1995, the placebo and Doxorubicin (15mg/kg) group (N=25) had a mortality rate of 32% where animals had enlarged abdomens, and presented as weak and lethargic.[119] In the same study, a group receiving Probucol (120mg/kg) combined with Doxorubicin (N=25) had preserved survival with a mortality rate of 0%.[47, 119]

Consistent with previous studies by Singal and colleagues,[23, 36-40, 44-47, 119] prophylactic treatment with Probucol improved survival from acute treatment with Doxorubicin in our study. A substantial shift in the survival curve was most observed in the group receiving Probucol with the combined treatment of Doxorubicin and Trastuzumab where it was 94% after 5 days and 63% after 10 days. As the shift in the mortality curve was greater in the combined treatment group than Doxorubicin only, this may suggest a dual action of Probucol against both Doxorubicin and Trastuzumab. This is consistent with previous studies suggesting that both Doxorubicin[47] and antiErbB2-induced cardiomyopathy[50] are prevented by the administration of an antioxidant.

4.5 Myocardial remodeling

Doxorubicin-induced cardiotoxicity can be clinically detected by invasive endomyocardial biopsy. Bristow et al. studied endomyocardial changes due to Doxorubicin (0-545 mg/kg) in 33 patients referred for symptoms of heart failure (N=7), research purposes (N=15), or advice for whether continuing therapy was safe (N=11).[123] The characteristic endomyocardial changes included myofibrillar loss and vacuolization due to distention of the sarcoplasmic reticulum. Further, histological examination of endomyocardial biopsy is sensitive to accurately assess the severity of Doxorubicin-induced cardiomyopathy using the Billingham scale.[44]

Similarly, rodent models confirm that histological examination of Doxorubicin-induced cardiomyopathy characteristically reveals extensive fibrosis, myofibrillar loss, scattered cardiomyocytes with vacuolar degeneration.[19] Siveski-Iliskovic et al. found histological evidence of cardiomyopathy in Doxorubicin treated mice (15 mg/kg, cumulative over two weeks).[36] Consistent with clinical biopsy observations Doxorubicin treated mice demonstrated swollen mitochondria and sarcoplasmic reticulum, vacuolization, formation of lysosomal bodies, and dilation of the sarcotubular system.[36] Higher magnification revealed loss of mitochondria cristae. The combination of Probucol (120mg/kg) with Doxorubicin prevented the histological evidence of Doxorubicin-induced cardiomyopathy. Using the same model, these findings have been replicated by three additional studies. A

comparison of Probucol treated rats to placebo receiving Doxorubicin reveals that cardiomyocytes are protected from increased vacuolization, fibrosis and overall cellular degeneration.[23] This was replicated by Kumar et al. 2001 in the examination of histological sections from Doxorubicin (15mg/kg) treated rats 21 days after six (2.5mg/kg) injections. Four days after treatment, cardiac sections revealed evidence of vacuolization.[35] This was substantially worse at 21 days here the purkinje cell layer in the subendocardial reagion was apparent as well as severe vacuolization.[35]

Characteristic histological findings have been observed in the setting of anti-HER2/ErbB2 therapy. The examination of cardiac sections from adult CKO HER2/ErbB2 mice demonstrated ventricular dilation, thinned walls and cardiomyocyte hypertrophy.[114] Additionally, the heart-to-body-weight ratios were increased and histological examination revealed enlarged T-tubules. Heart-to-body-weight ratios were also increased. Similar findings were found by Crone et al. 2002 in their histological examination of HER2/ErbB2-CKO mice where histological examination with transmission electron microscopy revealed increased mitochondria and vacuoles.[49] Riccio et al 2009 conducted a study comparing cardiotoxicity Trastuzumab in mice to other novel HER2 targeting antibodies.[124] The study used Erbicin-human-RNase (Erb-hrRNase), a fusedion of human pancreatic RNase with Erbicin and an engineered human anti-ErbB2-compact antibody (Erb-hcAb).[124] All antibodies in this murine model, including Trastuzumab, bound to mouse cardiomyocytes. Although Masson's trichrome

staining of immunoRNase and Erb-hcAB treated sections had increased cardiac fibrosis in comparison to control, it was significantly less than Trastuzumab and Doxorubicin.[124] Trastuzumab groups demonstrated greater cardiac fibrosis compared to control, where it was 13% versus 4%. Doxorubicin treated groups however had the highest amount of fibrosis where it was 17%.[124] Finally, Jassal et al. recently demonstrated that mice receiving D+T had the greatest degree of histological evidence of cardiomyocyte damage with extensive cellular vacuolization and clearing of cytoplasm.[40]

Similarly in our study, histological examination of placebo mice revealed extensive myofibular degeneration and vacuolization that was worse in the combination of D+T. Probucol attenuated this effect where there was minimal myofibular degeneration and vacuolization in doxorubicin and the combination of D+T. This is consistent with previous studies that probucol prevents cardiomyocyte degeneration as examined with Masson's trichome.[40, 52] It is novel that the prophylactic treatment of probucol prevents myofibular changes in the combination of Doxorubicin and Trastuzumab.

4.6 Apoptosis

The pathology of cardiac remodeling in the setting of Doxorubicin-induced cardiotoxicity has been suggested to be due to apoptosis. A previous study by Kumar et al 2001 evaluated Probucol's role in modulating apoptosis in

Doxorubicin-induced cardiomyopathy.[35] In this chronic rat model of chemotherapy, TUNEL staining confirmed apoptotic myocytes at day 4, which was progressively more severe at 21 days.[35] A comparison of the percentage of apoptotic cardiomyocytes at endpoint revealed a 5-fold increase in the Doxorubicin group. The administration of Probucol however significantly lowered the degree of Bax/Bcl-xl ratio by 3.5% relative to the D only group.[35] Bax protein expression was increased in the Doxorubicin group and Probucol was cardioprotective against apoptosis.[35] This study demonstrated that Doxorubicin-induced cardiomyopathy correlates with increased Bax expression and that Probucol lowered the Bax/Bcl-2 ratio in rat hearts.[35]

Similarly, Nielan et al. demonstrated that Doxorubicin-induced cardiomyopathy is mediated by apoptosis in an acute murine model of chemotherapy induced cardiac dysfunction.[52] Wild type mice that received a single dose of Doxorubicin (20mg/kg) showed severe cardiac dysfunction this was associated with increased expression of proapoptotic gene expression, Bax, and decreased antiapoptotic Bcl-XL expression.[52]

Mitochondrial dysfunction and altered Bax and Bcl-XL expression have been well established in rodent models of Trastuzumab-induced cardiomyopathy. Crone et al. found that there was increased apoptosis in the myocardium of HER2/ErbB2-CKO mice.[49] To determine whether apoptosis was a cause of cardiomyopathy, the authors confirmed that overexpression of the anti-apoptotic gene Bcl-XL was

cardioprotective. Moreover, in vivo expression of Bcl-XL partially rescued both chamber dilation and contractility in six week old ErbB2-floxed/MCK-Cre mice.[49]

Grazette et al. (2004) used a monoclonal antibody for the HER2/ErbB2 receptor in rats with a 40% binding specificity to cardiomyocytes.[51] A substantial decrease in the anti-apoptotic Bcl-XL protein expression was found in both neonatal and adult cardiomyocytes while pro-apoptotic Bax and Bcl-xS proteins were upregulated.[51] The increased Bcl-xS/Bcl-XL and Bax ratios were associated with cystolic cytochrome c, confirming the mitochrondrial pathway. TUNEL staining and propidium iodide flow cytometry confirmed increased percent apoptosis in anti-erbB2 treated cardiomyocytes. Furthermore, the administration of a cell permeable BH4, fused to the protein transduction domain of HIV-1 TAT protein (BH4-TAT), prevented apoptosis in this setting.[51] BH4-TAT is a peptide that acts by exerting antiapoptotic activity and inhibits overexpression of Bcl-XL.[125] Cell therapy with BH4-TAT was cardioprotective in the setting of anti-ErbB2 by facilitating the upregulation of anti-apoptotic Bcl-XL proteins.[51]

Similarly, Jassal et al. (2009) confirmed that the combination of Doxorubicin and Trastuzumab in an acute murine model of chemotherapy induces a higher degree of apoptosis than the administration of either drug alone.[40] Mice receiving Doxorubicin (20mg/kg) had elevated levels of antiapoptotic Bcl-XL and decreased levels of proapoptotic Bax. Trastuzumab (10mg/kg) treated mice also had elevated Bax/Bcl-XL ratios in comparison to control. The combination of

Doxorubicin and Trastuzumab had the greatest degree of Bax/Bcl-XL protein expression 5 days after treatment.[40] This suggests that in an acute murine model, the combination of Doxorubicin and Trastuzumab is associated with greater proapoptotic protein expression than Doxorubicin alone.

Riccio et al. (2009) found decreased cell viability for Trastuzumab treated neonatal cardiomyocytes and H9C2 cardiomyoblasts.[124] The response was dose dependant where 0.1μM was less than control and 0.5μM of Trastuzumab had the equivalent percent cell viability as 0.1μM Doxorubicin.[124] A comparison of Bcl-XL/actin ratio demonstrated that there was minimal Bcl expression in both H9C2 cardiomyoblasts 72 hours after cell treatment with Trastuzumab in comparison to control.[124] The findings of Gordon et al. were similar showing that Trastuzumab-induced cardiomyopathy acts via mediating erbB2 signaling through a mitochondrial and Bax/Bak-dependent apoptotic pathway.[50] Bcl-XL expression is lower in Trastuzumab treated human cardiomyocytes.

Our findings supports altered protein expression in mice treated with either Doxorubicin and/or Trastuzumab. Consistent with previous studies, Doxorubicin (20mg/kg) treated mice had an increase in Bax expression with decreased Bcl-XL expression. Prophylactic Probucol treatment with Doxorubicin lowered the Bax/Bcl-XL ratio, as previously shown in chronic rodent models. As expected from antiErbB2 models, Trastuzumab treated mice also had increased Bax/Bcl-XL ratios. A novel finding that Probucol lowered the Bax/Bcl-XL ratio in

Trastuzumab treated mice suggests that Trastuzumab-induced cardiomyopathy is prevented by antioxidants. Moreover, a 5-fold increase in Bax/Bcl-XL from the combined treatment of Doxorubicin and Trastuzumab was prevented by Probucol. This suggests the administration of Probucol is associated with a decrease in the degree of apoptosis from Trastuzumab, Doxorubicin, or the combination of D+T, as expressed by a lower Bax/Bcl-XL ratio.

4.7 Clinical implications

The application of Probucol as a cardioprotective drug against Doxorubicin and Trastuzumab-mediated cardiomyopathy is a novel and clinically applicable finding. Although dexrazoxane, ACEI, β-blockers and ARB's may be useful in the setting of Doxorubicin-induced cardiomyopathy, no previous studies have evaluated their use in the prevention of Doxorubicin and Trastuzumab-induced cardiomyopathy. Our current study is novel in suggesting that Probucol is an effective pharmacological agent in preventing Trastuzumab-induced cardiotoxicity. Although other antioxidants including N-acetylcysteine may prove useful in this setting, further clinical investigation is required.

4.8 Conclusion

Our novel study demonstrates, for the first time, that prophylactic therapy with the antioxidant Probucol preserves systolic function and mortality in mice treated with Doxorubicin and Trastuzumab. Further research is required for the translation of prophylactic antioxidant therapy into the clinical setting of patients receiving Trastuzumab for HER2 overexpressed breast cancer.

4.9 Future Studies

Although we have successfully established an acute murine model of chemotherapy induced cardiotoxicity and have examined the prophylactic role of Probucol in its prevention, a chronic murine model emulating the clinical setting is warranted. Future studies will evaluate the role of antioxidants, renin angiotensin aldosterone antagonists and beta blockers in the prevention of doxorubicin and trastuzumab mediated cardiotoxicity in a chronic murine model with translational application to the clinical arena.

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Appendix A

Probucol was previously indicated for use adjunct to dietary therapy for decreasing elevated serum total and LDL-cholesterol concentrations in the setting of primary hypercholesterolemia. Clinical contraindications included ventricular arrhythmias or hypersensitivity to Probucol. Drug interactions involve: 1) the risk of enhancing QTc-prolonging effects with Alfuzosin, Artemether, Chloroquine, Ciprofloxacin, Ciprofloxacin, Dronedarone, Gadobutrol, Lumefantrine, Nilotinib, Pimozide, QTc-Prolonging Agents, QuiNINE, Tetrabenazine, Thioridazine, Ziprasidone; and, 2) the risk of decreasing serum concentration of CycloSPORINE. Adult dosing for dyslipidemia was 500mg twice daily with morning and evening meals; and, 250mg QID for pediatics less than 27Kg or 500mg QID for pediatics greater than 27Kg. Probucol acted by increasing the fecal loss of bile bound LDL cholesterol via inhibiting the enteral cholesterol absorption and decreasing the synthesis of cholesterol.