Early Detection of Broken Hearts in Cancer:

Bevacizumab and Sunitinib Mediated Cardiotoxicity

by

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Abstract

Background: Although Bevacizumab (BVZ) and Sunitinib (SNT) prolong survival in cancer patients, an unanticipated side-effect is cardiotoxicity. Early indices of left ventricular (LV) systolic dysfunction would be useful to address the cardiac safety of anti-cancer drugs.

Objective: Whether cardiac biomarkers, tissue velocity imaging (TVI), and/or strain rate (SR) can detect early cardiac dysfunction.

Methods: A total of 95 C57Bl/6 mice received one of the following drug regimens: i) 0.9% saline; ii) BVZ; or iii) SNT and followed for 14 days. Serial blood pressure, high sensitivity troponin I (hsTnI), and echocardiography were performed.

Results: BVZ- and SNT-treated mice demonstrated an increase in mean arterial blood pressure, hsTnI, cardiac apoptosis, and loss of cell integrity. TVI and SR values confirmed early LV systolic dysfunction at day 8, compared to conventional LVEF at day 13.

Conclusions: Novel imaging techniques can detect early LV systolic dysfunction in a model of drug-mediated cardiomyopathy.

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List of Abbreviations

ACS	Acute coronary syndrome
ADCC	Antibody-dependent cell-mediated cytotoxicity
AMP	Adenosine monophosphate
AMPK	AMP-protein activated kinase
ATEs	Arterial thrombotic events
ATP	Adenosine triphosphate
BEV-CAPIRI	Bevacizumab, capecitabine and irinotecan
bFGF	Basic fibroblastic growth factor
BMI	Body mass index
BNP	Brain natriuretic peptide
BVZ	Bevacizumab (Avastin)
CAD	Coronary artery disease
CDC	Complement-dependent cytotoxicity
CHF	Congestive heart failure
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CSF-1R	Colony stimulating factor receptor type 1
CT	Computed tomography
CTCAE	Common Terminology Criteria for Adverse Events
DOX	Doxorubicin
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal-regulated kinase
FABP	Fatty acid binding protein
FAK	Focal adhesion kinase
FDA	Food and Drug Administration (US)
FLT3	Fms-like tyrosine kinase 3
FOLFOX4	5-FU, leucovorin, and oxaliplatin
FS	Fractional shortening
gFOBT	guaiac fecal occult blood test
GAPDH	Gluceraldehyde-3-phosphate dehydrogenase
GIST	Gastrointestinal stromal tumor
GPBB	Glycogen phosphorylase isoenzyme BB
hERG	Human ether-a`-go-go- related gene
HER-2	Human epidermal growth factor receptor 2
HF	Heart failure
HIF-α	Hypoxia inducible factor-alpha
HR	Heart rate
hsTnI	High sensitivity troponin I
IFL	Irinotecan, fluorouracil, and leucovorin
IFN-α	Interferon alfa
Ig	Immunoglobulin
IL-2	Interleukin 2
KIT	Stem cell factor receptor

LV	Left ventricle
LVEF	Left ventricular ejection fraction
$LVID_{ED}$	Left ventricular end-diastolic diameter
$LVID_{ES}$	Left ventricular end-systolic diameter
MAP	Mean arterial blood pressure
MAPK	Mitogen-activated protein kinase
mCRC	Metastatic colorectal cancer
MEK	Mitogen-activated protein kinase kinase
MI	Myocardial infarction
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MUGA	Multiple-gated acquisition
NCI	National Cancer Institute
NO	Nitric oxide
NOS	Nitric oxide synthase
OS	Oxidative stress
PAI-1	Plasminogen activator inhibitor
PARP	Poly ADP ribose polymerase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PET	Positron emission tomography
PI3K	Phosphoinositide 3’-kinase
PKC	Protein kinase C
PLC- γ	Phospholipase C-γ
PlGF	Placental growth factor
pRCC	Hereditary papillary RCC
PWT	Posterior wall thickness
RCC	Renal cell carcinoma
RET	Transfection receptor kinase
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SNT	Sunitinib (Sutent)
SRI	Strain rate imaging
SR	Strain Rate
TAC	Transverse aortic constriction
TDI	Tissue Doppler imaging
TKIs	Tyrosine kinase inhibitors
TNF-α	Tumor necrosis factor alpha
TnI	Troponin I
TNM	Tumor, node, and metastasis
TnT	Troponin T
t-PA	Tissue type plasminogen activator
TRZ	Trastuzumab
TTE	Transthoracic echocardiography
US	Ultrasound

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
V_{endo}	Peak endocardial systolic velocity
VHL	Von Hippel-Lindau syndrome
VPF	Vascular permeability factor
5-FU	5-fluorouracil

Chapter 1: Literature Review

Cancer in Canada: Introduction

Cancer is a major public health issue in Canada and is the second leading cause of death next to cardiovascular disease. Each day, over 500 Canadians are diagnosed with cancer and 200 die from the disease.[1] In 2013, approximately 187, 600 new cases and 75, 500 deaths from cancer were reported,[2] which is a 1% annual increase.[2] National mortality rates indicate that 24% of women and 29% of men, or 1 in 4 Canadians will die of cancer in their lifetime.[2] Men are more affected by cancer than women and the disease risk increases with age, irrespective of sex. In 2013, 71% of new cases and 83% of deaths occurred in individuals over the age of 60.[2] Combined, colorectal and renal cell cancers account for nearly 1/5 of all cancers diagnosed among men and women in Canada annually.[2]

Cardio-Oncology

Cardio-Oncology is an emerging discipline that involves the management of cancer patients who experience cardiovascular complications as a result of cancer treatment. In the last half century, the use of potent chemotherapeutic agents and radiotherapy for the treatment of cancer has significantly improved mortality outcomes. However, the use of traditional chemotherapeutic agents, including anthracyclines such as Doxorubicin (DOX), increase the risk of developing heart failure (HF) by 5% in cumulative doses of $\geq$ 400 mg/m^2 and by 25% in cumulative doses above 550 mg/m^2.[3-5] Current oncological studies demonstrate that among patients exposed to chemotherapy, more than half exhibit some degree of cardiac dysfunction 10-20 years post-treatment, while 40% experience cardiac arrhythmias.[6] Recently, an improved understanding of the biochemical

mechanisms of cancer and tumor cell progression has led to the development of targeted drugs, which act through inhibition of specific target molecules. Although these agents are more effective in the cancer setting, a consequence in the use of targeted agents is the development of cardiotoxicity.[7] As the awareness of the cardiotoxic side-effects caused by these agents improves, it is imperative that cardiologists, oncologists, and basic scientists collaborate to better predict patient susceptibility to drug-mediated cardiac dysfunction. In addition, strategies are needed to provide long-term follow-up care for patients and to ensure successful treatment of the disease, while reducing the occurrence of cardiovascular events.[8-10]

Drug-mediated cardiotoxicity has been defined by the Cardiac Review and Evaluation Committee evaluating Trastuzumab clinical trials, as being one or more of the following: a) cardiomyopathy in terms of a reduction in left ventricular ejection fraction (LVEF); b) symptoms associated with congestive heart failure (CHF); c) signs associated with CHF, such as tachycardia; d) reduction in LVEF between $\geq$ 5% to $\leq$ 55% from baseline, with accompanying signs or symptoms of HF, or a reduction in LVEF ranging from $\geq$ 10% to $\leq$ 55%, without accompanying signs or symptoms.[11] Cardiotoxicity is a growing problem in the oncological setting due to the increasing number of long-term cancer survivors, use of progressively higher doses of anthracyclines, and the introduction of new anti-tumor agents in combination with various treatments, resulting in harmful cardiovascular side effects.[12-15] This phenomenon was first observed with the combined administration of DOX and Trastuzumab (TRZ). TRZ is a monoclonal antibody targeted against the extracellular domain of the human epidermal growth factor receptor 2 (HER-2) protein which is overexpressed in a subset of breast cancers.[16-18] The

use of DOX+TRZ in the adjuvant setting resulted in a 50% decrease in the risk of breast cancer relapse and a 33% decrease in breast cancer mortality.[19-21] However, TRZ augments the cardiotoxic side effects of DOX and results in a 25% risk of developing drug induced cardiomyopathy.[22, 23] As a result, alternative treatment therapies have been investigated for the safe and effective treatment of breast cancer and various other malignancies.

Increased understanding of the mechanism underlying tumor cell progression has lead to the development of novel drugs that inhibit angiogenesis by targeting specific pro-angiogenic factors.[24] Two of these drugs include Bevacizumab and Sunitinib, which target vascular endothelial growth factor (VEGF) protein signaling and tyrosine kinase receptors, respectively. Although effective in inhibiting the VEGF signaling pathway, it has become increasingly apparent over the last several years that these novel anti-cancer drugs are also associated with an increased risk of cardiotoxicity, including hypertension, vascular events, and cardiomyopathy.[25]

Colorectal Cancer: Prevalence, Diagnosis, and Treatment

Colorectal cancer is a major public health concern for Canadians as it is the third leading cause of cancer related illness and cancer related deaths among men and women.[2] In 2013, it is estimated that more than 23,300 new cases and 9,200 deaths from colorectal cancer will be reported in Canada.[2] The mortality rate of colorectal cancer equates to 12% of all cancer deaths per annum.[2] An estimated 72% of cases arise in the colon and approximately 28% originate in the rectum.[26] Throughout a lifetime, men and women share an equal risk of developing colorectal cancer, approximately 5% or 1 in 20

chance.[26] The American Cancer Society estimates that 143,000 new cases of colorectal cancer will be reported in 2013, resulting in 51,000 deaths in the United States.[27]

The diagnosis of colorectal cancer commonly does not occur until the progressive disease stage. This delayed diagnosis can be attributed to the nonspecific nature of physical symptoms. However, the identification of lesions prior to the onset of metastases results in significantly reduced mortality.[28] A variety of pathophysiologic signs and symptoms are associated with advanced colorectal cancer, including: i) change in normal bowel pattern; ii) diarrhea/constipation; iii) abdominal pain/cramping; iv) rectal bleeding; and v) the presence of hypochromic, microcytic anemia.[29] Several etiologic factors, such as increasing age, are associated with an increased risk of developing colorectal cancer. Men and women experience a greater risk after the age of 40, with the relative risk of colorectal cancer doubling each decade after 50 years of age.[28] Predisposing genetic factors including, familial polyposis coli and Lynch syndrome, are associated with a significant proportion of colorectal cancers.[30] In addition, patients with a previous medical history of colorectal cancer, ulcerative colitis, or severe dysplasia are at greater risk of developing colorectal neoplasms.[31] Considering that physical abdominal examinations reveal very little about the presence of colorectal tumors, more sensitive diagnostic procedures have been developed for the detection of colorectal malignancies.

Advanced screening and early detection are key in the diagnosis and primary treatment of colorectal cancer. The main barriers perceived by Canadians when considering colorectal screening include the personal lack of need to be tested and decreased awareness of preventative screening procedures. As patients are further educated on the importance of preventative colorectal screening, the disease can be

detected in its early stages of progression, translating to survival rates ≥ 90%.[32] In 2011, the Canadian Colon Cancer Screening survey demonstrated that 50% of Canadians aged 50-74 years were up-to-date with their screening tests, an increase from 44% of Canadians in 2009.[33] Screening procedures including the guaiac fecal occult blood test (gFOBT) and colonoscopy, performed every two years and five years respectively, have been shown to reduce incidence and overall mortality of the disease.[34-37] National screening recommendations for colorectal cancer have been in place in Canada for the past 10 years[38, 39], which led to the adoption of screening or pilot programs by all provinces in 2010. Manitoba, one of the first provinces to launch the colorectal cancer screening program in 2007, offers screening for > 80% of the population.[33]

Treatment of colorectal cancer is multifaceted, including surgery, radiation therapy, fluorouracil-based chemotherapy, and the recent addition of novel monoclonal antibodies. In the presence of advanced colorectal cancer, primary tumor resection is considered as first line management in the majority of cases.[40] A surgical approach for colorectal cancer is dependent on tumor stage and localization[41], and includes polypectomy and local excision, anterior resection of the rectum, and open colectomy whereby a segment of the colon and nearby lymph nodes are removed. Laparoscopic resection, although less invasive than open colorectal resection, remains to be widely implemented due to its complexity and increased operative times.[42] Radiation therapy used in combination with surgical and chemotherapy treatments has demonstrated beneficial health outcomes and reduced recurrence rates.[43, 44] In the neoadjuvant setting of colorectal cancer, radiation therapy plus the addition of a chemotherapeutic and targeted agent resulted in tumor regression and 5-year disease free-survival in 75% of

patients.[45] Multimodality treatment of colorectal improves overall survival when compared to chemotherapy alone (46.0 months vs. 20.2 months, p=<0.0001).[46] The role of chemotherapy against colorectal cancer has expanded in the last decade and includes the combination of 5-fluorouracil (5-FU), leucovorin, oxaliplatin, and the addition of a molecular targeted agent.[47]

Monoclonal Antibodies: History of Bevacizumab

The theory of targeting angiogenesis as an anti-cancer strategy was first proposed by Folkman over 40 years ago.[48] Several monoclonal antibodies have been produced for use in the clinical setting and are shown to demonstrate high purity, predefined specificity and good reproducibility, as compared to polyclonal antibodies.[49] The first monoclonal antibody drug to be approved by the US Food and Drug Administration was muromonab in 1986, which was used as therapy for patients experiencing transplant rejection. In the last decade, monoclonal antibodies have become known as an effective pharmacotherapy and are implemented in the treatment of a wide variety of diseases, ranging from autoimmune disorders to cancer.

Approximately half of all monoclonal antibodies can be categorized as anti-cancer agents[50] and have demonstrated efficacy in cancer treatment. Monoclonal antibodies are designed with a high specificity for antigens expressed on tumor cells and their development includes three separate stages: i) murine antibodies; ii) rat/human chimeric antibodies; and iii) humanized antibodies.[51] Murine antibodies were initially used but were viewed as foreign by the human immune system, and thus were modified to reduce immunogenicity.[52] Humanized antibodies are created by modifying the protein sequences of non-human antibodies to increase their similarity to antibody variants produced

naturally in humans.[53] There are five classes of immunoglobulins, including; IgA, IgD, IgE, IgG, and IgM. The majority of clinical therapeutic antibodies are of the human IgG1 isotope and demonstrate a relatively long half-life of 21 days.[54] Bevacizumab, a recombinant humanized antibody belonging to the IgG immunoglobulin class, was the first approved therapeutic agent to specifically target the vessels of solid colon, lung, and breast tumors.[55]

Bevacizumab Anti-cancer Mechanisms

Several theories have been proposed to explain the therapeutic efficacy of monoclonal antibodies in decreasing solid tumor size. These mechanisms include: i) modulation of the receptor; ii) antibody-dependent cell-mediated cytotoxicity (ADCC); iii) complement-dependent cytotoxicity (CDC); and iv) induction of apoptosis.[56-61] Several studies have demonstrated that ADCC plays a central role in the effectiveness of therapeutic antibodies, depending on the presence of antigen-coated target cells and Fc gamma receptor positive effector cells.[62, 63]

Angiogenesis, which is the formation of new blood vessels, is essential in embryonic development, tissue regeneration, and wound healing. Angiogenesis has become widely recognized as an important therapeutic target in metastatic colorectal cancer (mCRC), as well as many other forms of cancer.[64] Tumors require an extensive network of blood vessels to supply nutrients and oxygen and to ensure their growth and metastasis, which is known as tumor angiogenesis.[48] In order for a primary tumor to acquire this capacity, an 'angiogenic switch' must be activated, whereby an imbalance is created between pro-angiogenic and anti-angiogenic factors.[65] As tumors increase in size, hypoxia-inducible factor-1α is produced, which leads to increased VEGF transcription.[66] Secreted VEGF

binds to endothelial cell receptors and elicits several responses, including: i) increasing microvascular permeability[67]; ii) activating matrix-degrading enzyme secretion; and iii) stimulating endothelial cell-proliferation, migration, and survival.[68] Additionally, hypoxia also results in increased production of various pro-angiogenic molecules including nitric oxide synthase, platelet-derived growth factor (PDGF), and basic fibroblastic growth factor (bFGF). However, VEGF has been deemed to be most critical of these factors.[69, 70] The augmented production of VEGF causes increased vessel permeability and endothelial cell proliferation, resulting in cell adhesion and lumen formation. In contrast to normal vasculature, neoplastic blood vessel formation is characterized by structural and functional abnormalities, in addition to the dysregulated expression of angiogenic and anti-angiogenic factors.[71]

Angiogenesis inhibition can occur either through direct or indirect mechanisms. Direct angiogenic inhibition results from local suppression of endothelial cell proliferation and migration mediated by endogenous proteins that include endostatin, angiostatin, and tumstatin.[72] In contrast, indirect angiogenic inhibition occurs through the neutralization of ligands such as VEGF and PDGF and the blockade of receptor tyrosine kinases including VEGF receptor (VEGFR) and PDGF receptor (PDGFR).[73] Indirect anti-angiogenic agents more commonly induce drug resistance due to their targeting of unstable tumor cells rather than genetically stable endothelial cells.[72, 74] In recent years, the growth factor VEGF, also known as VEGF-A, has become recognized as a major angiogenic factor that is primarily involved in regulating tumor angiogenesis[75] and normal vascular development.[76] VEGF-A belongs to a supergene family of growth factors that include VEGF-B, VEGF-C, VEGF-D, PDGF, and placental growth factor

(PlGF). VEGF signals mainly through VEGF receptor 2 (VEGFR-2), which is highly expressed by endothelial cells engaged in angiogenesis and by circulating bone marrow-derived endothelial progenitor cells. The action of VEGF can be attributed to both paracrine and autocrine mechanisms.[77, 78] VEGF receptor activation leads to signaling via the Ras/Raf/MEK/ERK pathway, ultimately resulting in endothelial activation but cardiomyocyte injury. An additional mechanism of anti-angiogenic action involves VEGFR activation which triggers intracellular signaling by the phosphorylation of proteins including phospholipase C-γ (PLC- γ), focal adhesion kinase (FAK), p38, and phosphoinositide 3'-kinase (PI3K) (Figure 1).

Additionally, through normalization, which is the selective elimination of poorly formed blood vessels, VEGF inhibitors produce a dichotomous increase in tumor blood flow and oxygenation.[70] This normalized blood flow results in the enhanced delivery of cytotoxic chemotherapy and radiation therapy to the neoplastic tissue. Also, it has been demonstrated that tumor oxygenation is required for the delivery of radiation beams,[79, 80] allowing for a synergistic effect between radiation therapy and VEGF inhibitors. Due to the fact that angiogenesis inhibitors act through different mechanistic pathways and exert therapeutic effects apart from cytotoxic chemotherapy and radiation therapy, the combination of these therapies for the enhanced treatment of cancer is a reasonable treatment paradigm. Thus, anti-angiogenesis therapy via inhibition of the VEGF signaling pathway has become a widespread and effective antitumor strategy in current clinical practice.

Bevacizumab, known commercially as Avastin, is a monoclonal antibody that inhibits the pro-angiogenic growth factor VEGF in healthy and neoplastic tissues.[81-83]

The binding of Bevacizumab to VEGF-A leads to reduced small vessel growth, inhibition of new vessel formation, and decreased blood supply to the tumor.[84-88] (Figure 2). Bevacizumab is a humanized monoclonal antibody derived from murine anti-human VEGF and is 93% human and 7% murine.[89] In the presence of decreased VEGF signaling, malignant cells exhibit a decreased intracellular pressure, which results in greater vulnerability to chemotherapy and radiotherapy.[84] Bevacizumab was first approved by the United States Food and Drug Administration (FDA) in February 2004 for the treatment of metastatic colorectal cancer. Throughout the last decade, the use of Bevacizumab has expanded to other malignancies, including breast cancer, pancreatic cancer, prostate cancer, non-small cell lung cancer, metastatic renal carcinoma, and ovarian tumors.[55]

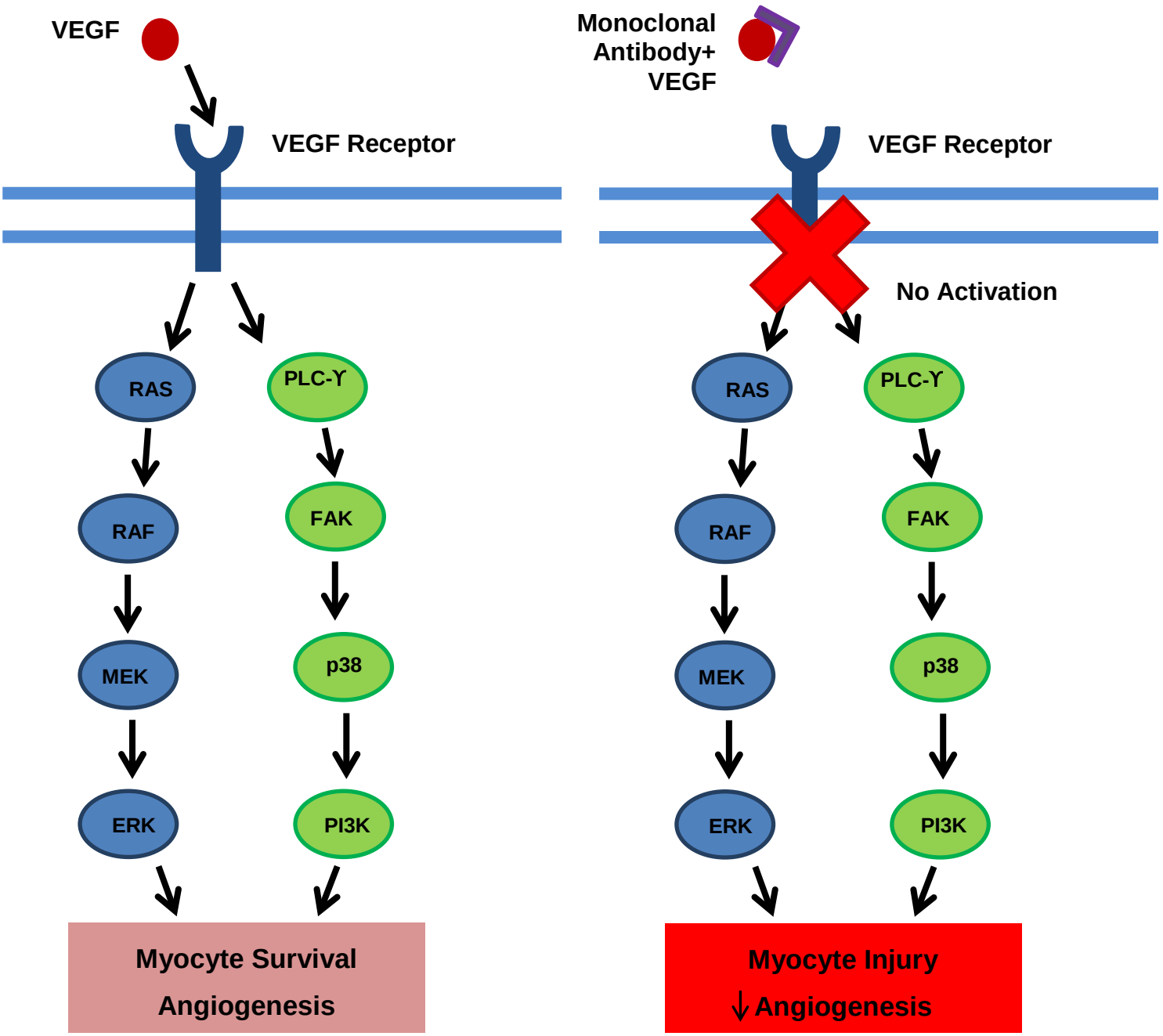


Figure 1: Anti-angiogenic mechanisms induced through the targeting of VEGF receptor. Vascular endothelial growth factor, VEGF; Mitogen-activated protein kinase kinase, MEK; Extracellular-singal-regulated kinase, ERK; Phospholipase C-γ, PLC- γ; Focal adhesion kinase, FAK; Phosphoinositide 3'-kinase, PI3K.

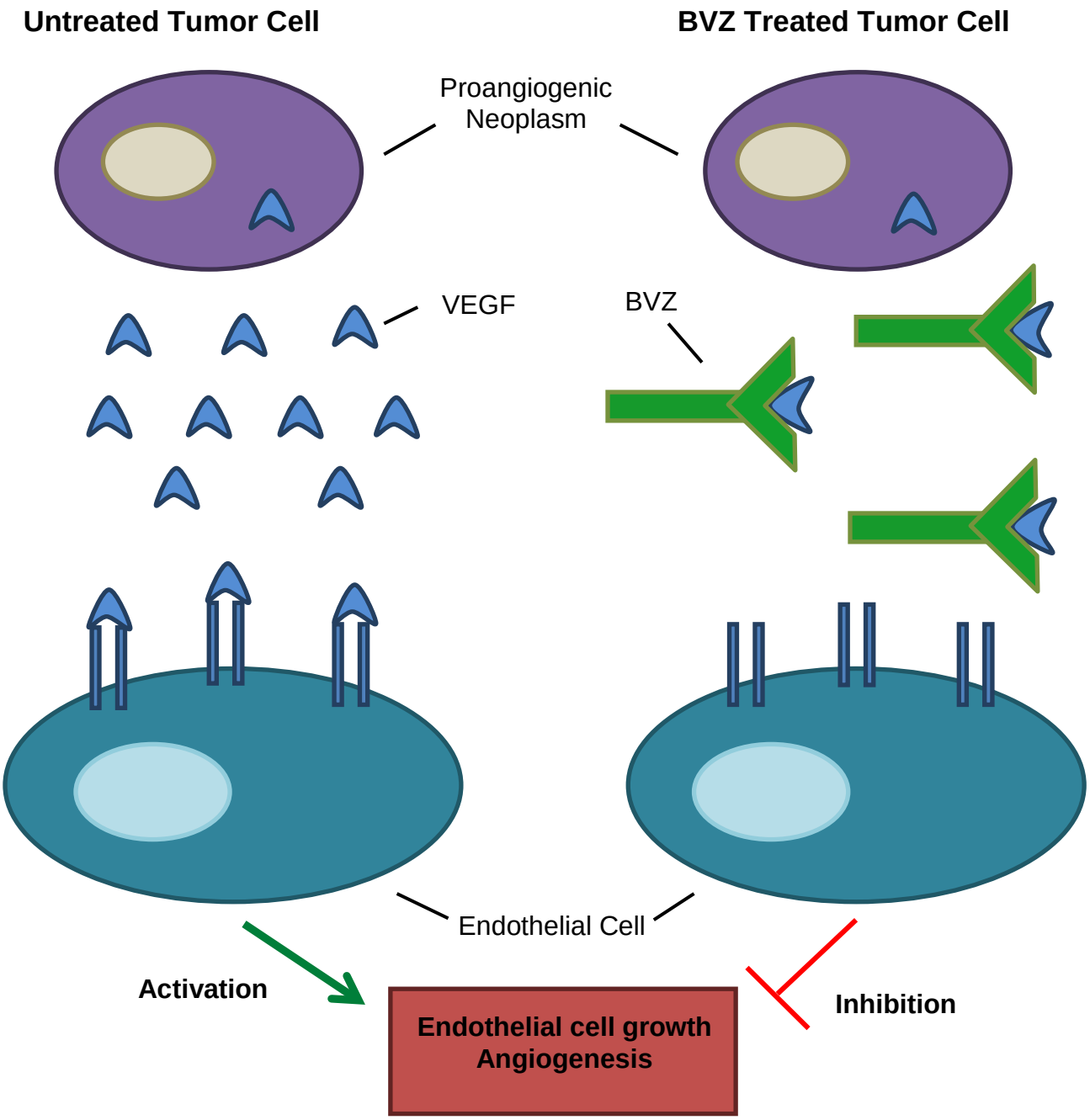


Figure 2: Mechanism of action of Bevacizumab (BVZ). VEGF normally binds to its receptor (VEGFR) on endothelial cells to promote endothelial proliferation and angiogenesis. The monoclonal antibody BVZ binds to VEGF inhibiting interaction with VEGFR and endothelial proliferation. Vascular endothelial growth factor, VEGF.

Bevacizumab-induced Cardiotoxicity

Bevacizumab is a novel targeted therapy that is used in the adjuvant cancer setting as well as in patients with stage III unresectable and stage IV advanced colon or rectal adenocarcinoma.[90, 91] In the past decade, the use of Bevacizumab has become widely accepted as an effective first-line therapy in the management of advanced colorectal cancer when combined with other conventional chemotherapy agents, including 5-florouracil (5-FU).[92-96] In 2006, the combined regimen of Bevacizumab, 5-FU, leucovorin, and oxaliplatin (FOLFOX4) was approved by the FDA as a second-line treatment for metastatic carcinoma of the colon or rectum. Bevacizumab is commonly administrated at a dosage of 5 mg/kg of body weight every two weeks for five consecutive cycles. Traditional chemotherapy agents delivered concurrently include 5-FU, leucovorin, oxaliplatin, and irinotecan. The chemotherapy regime is administered through bolus intravenous injection, which initially lasts 90 minutes and is subsequently reduced to 60, and 30 minutes with progressive treatments.

The integrated use of Bevacizumab with traditional chemotherapy agents in the advanced cancer setting has resulted in improved treatment response rates, progression free survival, and overall survival when compared to chemotherapy alone.[82, 83, 97] Hurwitz *et al.* evaluated the use of Bevacizumab plus irinotecan, fluorouracil, and leucovorin (IFL) for the treatment of metastatic colorectal cancer. Median duration of overall survival was significantly longer in patients given Bevacizumab and IFL as compared to those treated with IFL and placebo (20.3 months vs. 15.6 months), resulting in a 34% reduction in the risk of death in the Bevacizumab group.[83] In addition, the administration of Bevacizumab with IFL was associated with increased duration of progression-free

survival (10.6 months vs. 6.2 months) and increased response rate (44.8% vs. 34.8%).[83] Degirmenci and colleagues demonstrated that Bevacizumab plus capecitabine and irinotecan (BEV-CAPIRI) was superior to chemotherapy alone.[97] In patients who received BEV-CAPIRI as first-line treatment, median overall survival and progression-free survival was 25.3 months and 16.2 months respectively, as compared to 15.2 months and 10.2 months in patients given chemotherapy alone.[97]

Despite its demonstrated efficacy in the cancer setting, Bevacizumab treatment is associated with several severe adverse side-effects including thrombosis, arterial hypertension, proteinuria, perforation of the gastrointestinal tract, wound healing abnormalities, irreversible leuco-encephalopathy syndrome, allergic skin rash and hypersensitivity reactions.[88, 98, 99] More importantly, Bevacizumab has shown the potential to induce cardiovascular cardiotoxicity and irreversible heart failure in this patient population. Through decreased capillary permeability, pressure load is increased, which leads to cardiac hypertrophy and ultimately congestive heart failure.[100] In addition, Bevacizumab mediated cardiotoxicity is thought to occur via two key mechanisms, which include: i) alteration in nitric oxide (NO) production within the endothelium; and ii) impairment of myocardial angiogenesis as shown in Figure 3.[72, 87, 101-103] (Figure 3). A myriad of literature demonstrates that in both the basic science and clinical settings, Bevacizumab is associated with the development of hypertension and left ventricular (LV) systolic dysfunction.[87, 101-108]

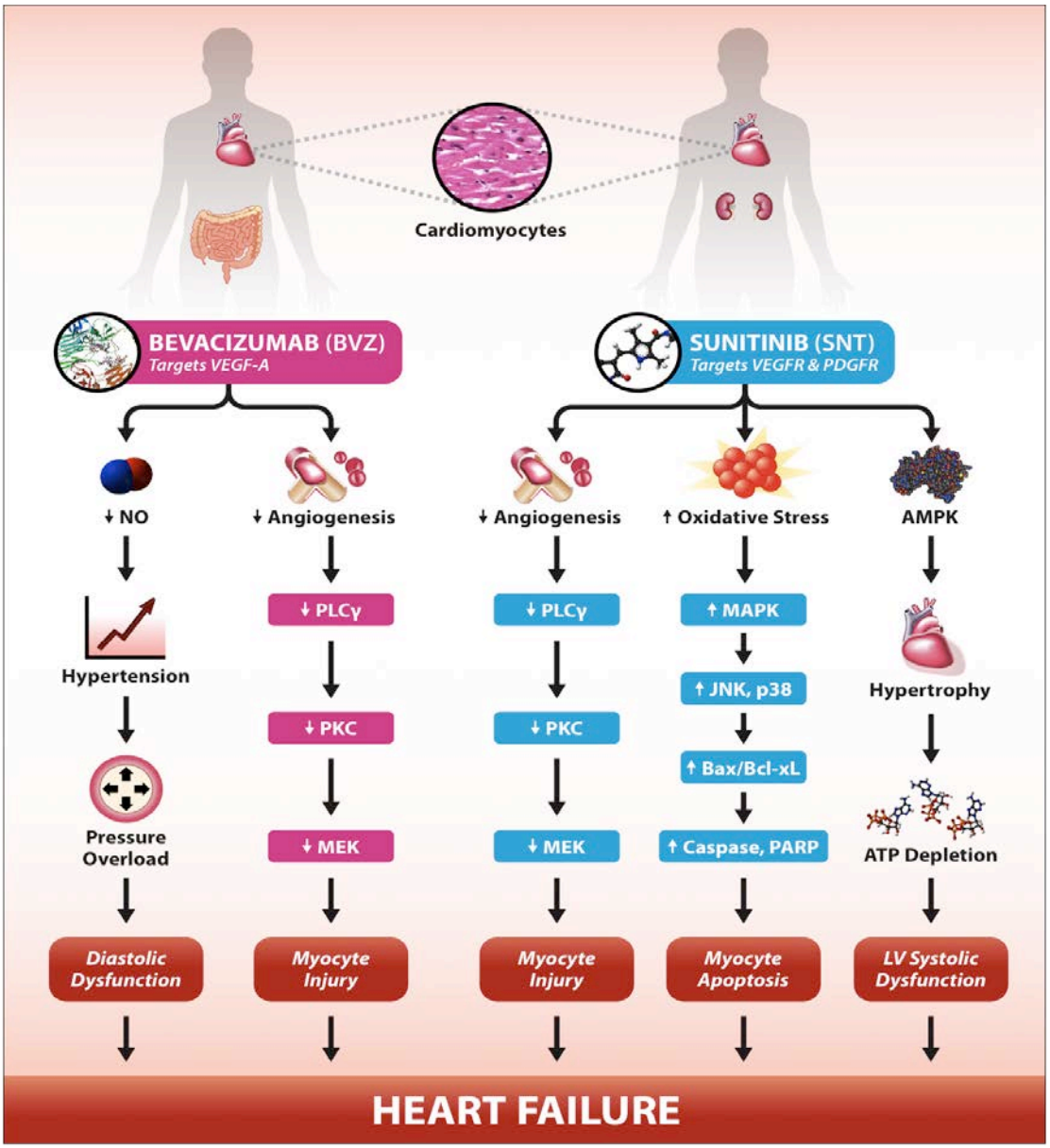


Figure 3: A schematic diagram illustrating the pathogenesis of Bevacizumab and Sunitinib mediated cardiotoxicity. Vascular endothelial growth factor, VEGF; Nitric oxide, NO; Phospholipase C-γ, PLC- γ; Protein kinase C, PKC; Mitogen-activated protein kinase kinase, MEK; Mitogen-activated protein kinase, MAPK; Poly ADP ribose polymerase, PARP; AMP-protein activated kinase, AMPK; Platelet-derived growth factor receptor, PDGFR; Vascular endothelial growth factor receptor, VEGFR.

i) Basic Science Model

A multitude of animal studies have demonstrated the harmful cardiotoxic side effects of Bevacizumab. A study done by Chen and colleagues found that mice treated with a combination of Bevacizumab and 5-FU demonstrated severe left ventricular systolic dysfunction, increased levels of troponin-I indicative of cardiomyocyte damage, and cardiac fibrosis.[87] In this animal model, once tumors reached 3-5 mm in diameter, mice were treated with 10 mg/kg of Bevacizumab once every 2 weeks through tail vein injection. In addition, Bevacizumab treated mice were administered 15 mg/kg of 5-FU intraperitoneally once per week, similar to the dosage delivered in the clinical setting. Animals were followed for between 10-20 weeks, in two separate experiments for the assessment of acute and chronic drug-induced cardiotoxicity.

In several animal models, VEGF has been shown to stimulate the production of NO and prostacyclins, which are responsible for the maintenance of systemic blood pressure, vascular remodeling, and angiogenesis.[101-103] Bevacizumab inhibits the VEGF signaling pathway and also reduces NO production, which leads to: i) development of hypertension; ii) pressure overload; iii) reduced myocardial capillary density; iv) global contractile dysfunction; v) cardiac fibrosis; and vi) subsequent heart failure in a murine setting.[24, 87, 101-104, 109] Furthermore, due to VEGF's important role in myocardial angiogenesis, mice with cardiomyocyte specific deletion of the VEGF gene demonstrate several abnormalities, including: i) fewer coronary micro-vessels within the heart; ii) thinning of the ventricular walls; iii) depressed basal contractile function; iv) induction of hypoxia-responsive genes involved in energy metabolism; and v) an abnormal response to β-adrenergic stimulation.[103] Finally, in a study by Neagoe *et al*., Bevacizumab induced

inhibition of VEGF-A led to decreased myocardial angiogenesis, decreased activities of PLCγ, PKC, and MEK, which resulted in increased myocyte injury with the development of heart failure.[104]

ii) Clinical Setting

Bevacizumab is commonly used in the management of advanced colorectal cancer and remains the most widely used and well-studied drug among the known anti-angiogenic agents. Currently, Bevacizumab combined with novel targeted drugs, including irinotecan and capecitabine, is the most widely used regimen in metastatic colorectal cancer resulting in increased response rates.[83, 97, 110, 111] In additon, Bevacizumab is the first agent demonstrated to improve survival by 30%, in patients with metastatic colorectal cancer.[89] However, this novel targeted drug has the potential to induce cardiovascular injury, as several studies have demonstrated the development of new or worsening hypertension in 25-30% of BVZ-treated patients.[105-108] Several proposed mechanisms for the acute development of hypertension and thromboembolic events include: i) the direct suppression of NO-and prostacyclin mediated vasodilation; ii) increased hematocrit and blood viscosity induced by overproduction of erythropoietin; and iii) altered expression of receptors in the renin-angiotensin-aldosterone system.[105-108, 112]

Chronic VEGF inhibition causes capillary rarefaction, which results in a number of events, including: i) reduced microvascular endothelial cell survival; ii) decreased tissue microvessel density; iii) local thrombosis; iv) decreased vascular perfusion; v) endothelial cell apoptosis; vi) microvessel obliteration; and vii) an increase in systemic vascular resistance.[113] Evidence also exists to support an increased rate of thromboembolic events

among Bevacizumab treated patients, including deep vein thrombosis, pulmonary embolism, transient ischemic attack, and acute mesenteric ischemia.[114-116]

Treatment with Bevacizumab has been shown to result in various adverse cardiovascular outcomes. Hypertension is commonly seen to develop in Bevacizumab-treated patients, with a myriad of clinical trials reporting an incidence rate between 4%-35%.[83, 117-119] The use of Bevacizumab is associated with twice the risk of developing stroke, myocardial infarction (MI), coronary artery disease (CAD), and cardiac death.[120] A recent clinical trial demonstrated that the incidence of heart failure in patients treated with Bevacizumab was 24 out of 1459, or 1.7%.[121] Comparatively, in a phase III clinical study of Bevacizumab-treated metastatic breast cancer, 2-3% of patients developed grade 3-4 heart failure or cardiomyopathy.[117, 118] Bevacizumab is also known to be associated with an increased risk of cardiac ischemia, although the reported prevalence of this adverse effect varies substantially from 0.5% to 2% across phase 3 randomized controlled trials.[122, 123] Another potential side effect seen in Bevacizumab treated patients is the occurrence of arterial thrombotic events (ATEs), including MI and angina.[121] A study by Scappaticci *et al*. that evaluated the use of Bevacizumab in a variety of settings, such as metastatic colorectal cancer, nonsmall cell lung cancer, and metastatic breast cancer, found that the overall incidence of ATEs to be 4%.[114] Clinical trials have reported that, although ATE events associated with Bevacizumab can occur at any time during therapy, the median time to event is approximately 3 months.[114, 124] Cumulative exposure and dosage are not predictors of event rate, however increasing age (>65 years) and prior ATE history have been identified as risk factors.[114, 124] Finally, in a retrospective clinical study by our group, from 2010-2011 at CancerCare Manitoba, evaluating the prevalence

of Bevacizumab mediated cardiac dysfunction, 14/76 (18%) colorectal cancer patients had LV systolic dysfunction.[125] Due to the multifactorial nature of cardiac disease progression identified in the setting of Bevacizumab, the relationship between cardiologists, oncologists, and basic scientists should be strengthened to ensure improved diagnosis and management of these adverse cardiovascular events that are associated with this targeted therapy.[126] In the novel field of Cardio-Oncology, techniques for the early detection of cardiac dysfunction are necessary for the prevention of end-stage heart failure in cancer patients treated with anti-angiogenic agents such as Bevacizumab.

Renal Cell Carcinoma: Prevalence, Diagnosis, and Treatment

In the twenty first century, renal cell cancer remains a national and international health issue. In 2013, approximately 5,900 new cases of renal cell cancer will be reported in Canada, translating to 1,750 deaths.[1, 2] As compared to various other malignancies, renal cell carcinoma (RCC) is the eighth most commonly diagnosed cancer in Canada, accounting for nearly 2.5% of all-cancer deaths.[2] The lifetime probability of developing renal cell cancer is 1 in 56 (2%) for men and 1 in 82 (1%) for women.[2] In Manitoba, when measured against all-cancer death rate, approximately 4% of men and 2% of women will die of renal cell cancer.[2] Renal cell cancer presents as a malignancy originating in the renal parenchyma and accounts for nearly 80% of all primary renal cancers.[127] Renal parenchymal cancers are comprised of clear cell carcinoma (70%), papillary (15%), chromophobe (5%), collecting duct cancers (1%), angiomyolipoma (1%) and other rare types (8%).[128] In the United States, it is estimated that approximately 65, 150 new cases of renal cell carcinoma will be reported in 2013, resulting in 13, 680 deaths nation wide.[27, 129]

Renal cell cancer is often diagnosed at an advanced disease stage due to several factors, which include: i) increasing tumor size in the absence of pain or other symptoms; ii) location of the kidney deep in abdomen prevents palpation on physical exam; and iii) lack of screening exams for patients who are not at increased risk of developing renal cancer. Etiologic factors that contribute to an increased risk of renal cell cancer include occupational exposure to asbestos and petroleum products, and those related to lifestyle, such as smoking, obesity, and hypertension.[130-132] In comparison to the general population, renal cell carcinoma is seen more commonly in patients with end-stage renal failure, acquired renal cystic disease, and tuberous sclerosis.[133, 134] However, about 2-3% of RCC cases are associated with familial and various autosomal-dominant syndromes.[135, 136] It has been demonstrated that the risk of RCC is more than double among individuals who have a first-degree relative diagnosed with kidney cancer.[136] Diseases which predispose patients to the development of hereditary tumors include: i) von Hippel-Lindau (VHL) syndrome (clear cell RCC); ii) hereditary papillary RCC (pRCC); iii) Birt-Hogg-Dube syndrome (chromophobe RCC); iv) hereditary leiomyomatosis; v) tuberous sclerosis; and vi) constitutional chromosome 3 translocation.[137] Of most significance is von Hippel-Lindau syndrome, which is characterized by alterations in the *VHL* gene and the development of several vascular tumors including clear renal cell carcinoma, haemangioblastomas of the central nervous system (CNS), pheochromocytoma, endolymphatic sac tumor, and papillary cystadenoma of the epididymis.[138] Genetic mutations in the *VHL* gene correspond to tumors which are early onset and multifocal in nature. Due to the relatively asymptomatic progression of

renal cell cancer, >50% of RCC cases are detected incidentally by noninvasive imaging examinations, for a variety of nonspecific symptoms or diseases.[139]

Early detection of renal cell carcinoma is possible through routine urinalysis and use of imaging techniques such as ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI). In clinical practice, the most commonly assessed laboratory parameters include serum creatinine, C-reactive protein, glomerular filtration rate, haemoglobin, erythrocyte sedimentation rate, alkaline phosphatase, and corrected serum calcium.[140] Determination of primary tumor extension, morphology of the contralateral kidney, lymph node involvement and degree of metastases are evaluated by abdominal and chest CT.[141] MRI may be indicated in the case of inconclusive CT results, contrast allergy, or pregnancy and provides information regarding renal mass, local tumor growth, and vena cava thrombus involvement. Positron emission tomography (PET) involves the assessment of tumor metabolism and may provide essential diagnostic information to clinicians, aiding in the diagnosis and management of cancer patients.[142] Another diagnostic tool is renal tumor biopsy, which is traditionally used before ablative and systemic therapy[143] as well as to differentiate benign from malignant renal tumors. Finally, severity of renal cell carcinoma can be assessed using the tumor, node, and metastasis (TNM) stage classification system, which enables clinicians to estimate prognosis of the disease. As there are currently no national screening procedures in place, Canadians at increased risk of developing renal cell carcinoma are encouraged to reduce exposure to tobacco, maintain a healthy weight and blood pressure, and visit their physician regularly.

Traditionally, treatment of renal cell carcinoma has involved surgical nephrectomy, ablation therapy, and the administration of interferon alfa (IFN-α) and/or interleukin 2 (IL-2). There are several factors that are considered prior to the initiation of RCC treatment, including tumor size, location of malignancy, extent of metastasis, renal function, existing comorbidities, and patient prognosis status. Surgical excision is the primary treatment for renal cell carcinoma, and includes radical nephrectomy, nephron-sparing partial nephrectomy, or laparoscopic nephrectomy. Conventional treatment with radical nephrectomy involves complete removal of the kidney with Gerota's fascia, the ipsilateral adrenal gland, and regional lymph nodes; however, less invasive procedures are now under investigation. Nephron-sparing partial nephrectomy is indicated for the treatment of tumors <4 cm in diameter, as well as bilateral renal masses, renal insufficiency, and in the presence of hypertension, diabetes, or hereditary renal-cell carcinoma syndrome. Survival results are similar between nephron-sparing and radical nephrectomies; however, the rate of local recurrence is greater with partial nephrectomy (3-6%).[139] Laparoscopic nephrectomy was first introduced in 1991[144] for the management of RCC, and serves as a minimally invasive surgical alternative to radical nephrectomy. Although laparoscopic partial nephrectomy is a technically difficult procedure, benefits from this technique include decreased postoperative pain, shorter hospitalization, and quicker patient recovery. Most recently, thermal ablative techniques that use radiofrequency heat ablation or cryoablation have been implemented for the treatment of RCC.[145, 146] Ablative treatment can be administered percutaneously or laparascopically;[147-149] however, laparascopic ablation is recommended when the tumor is in close proximity to adjacent organs. Limitations of thermal ablation include high local

recurrence rates compared with surgical excision,[150] poorly defined radiographic variables for success,[151] and difficulty in the successful ablation of tumors larger than 3.5 cm in diameter. Ideal candidates for percutaneous ablative therapy are patients of increasing age, who demonstrate substantial comorbidities, and do not qualify for conventional surgery.

Since the mid 1990's, IFN-α and IL-2 have been used for the systemic treatment of renal cell carcinoma, since RCC has shown a high resistance rate to conventional chemotherapy[152] and radiotherapy. The purpose of immunomodulatory therapy is to enhance either tumor antigenicity or host surveillance.[153] Exclusive use of IFN-α has shown increased response rates in approximately 14% of metastatic clear-cell renal carcinoma cases, with median duration of response between 6 and 24 months.[154] Standard therapy for advanced renal cell carcinoma includes high-dose IL-2 (600, 000 IU/kg i.v. every 8h for 14 doses, repeated once after a 9-day rest), which remains the only treatment regimen approved by the FDA. In the setting of renal cell carcinoma, low-dose IL-2 demonstrates lower response rates (13%) as compared to patients treated with high dose IL-2 (21%); however, both dosages are effective for a median of 54 months.[155] Unfortunately, high-dose IL-2 has been associated with several toxicities, most notably capillary leak syndrome, which limit its use.[156] In recent years, the systemic treatment of advanced RCC has grown to include several targeted agents, including sorafenib, temsirolimus, everolimus, pazopanib, and the tyrosine kinase inhibitor sunitinib.

Tyrosine Kinase Inhibitors: History of Sunitinib

Tyrosine kinase receptors, such as vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptors (PDGFRs), play important roles

in tumor angiogenesis.[24, 65] Unlike conventional chemotherapy agents, targeted therapies interfere with molecular targets that are involved with tumor growth and progression, providing a broader therapeutic potential with less toxicity. In the past, classic chemotherapy therapies including either 5-FU, paclitaxel, or DOX, have demonstrated low response rates (7%-12%) and have not impacted survival in the RCC population.[157] A possible explanation for the limited efficacy of chemotherapy is the high expression of p-glycoprotein in RCC cells, resulting in multidrug resistance.[158, 159] Thus, an increased understanding of the VEGF and mammalian target of rapamycin (mTOR) pathways, on which renal tumor cells rely, has lead to the discovery of relevant therapeutic targets. Additionally, the correlation between VHL syndrome and the development of clear cell RCC has lead to the investigation of the role of *VHL* gene inactivation.[160] Under normal conditions, *VHL* gene product regulates the protein degradation of hypoxia inducible factor-alpha (HIF-α) by forming stable complexes with elongin B, elongin C, cullin 2, and Rbx1.[161] In the absence of VHL protein, HIF-α accumulates and is free to bind with HIF-β[162] which forms a transcriptional factor complex leading to the ultimate transcription of hypoxia-inducible genes, including: i) VEGF; ii) epidermal growth factor receptor (EGFR); iii) PDGF; and iv) erythropoietin.[163] By binding to specific tyrosine kinase receptors, these growth factors promote tumor angiogenesis through increased cell migration, proliferation, and survival. Therefore, inhibition of VEGF and PDGF signaling pathways by tyrosine kinase inhibitors (TKIs) is an effective therapeutic approach in the setting of RCC.

Tyrosine kinases (TKs) are enzymes that catalyze the transfer of the γ phosphate group from adenosine triphosphate (ATP) to tyrosine residues in polypeptides. Tyrosine

kinases were first discovered as being oncogenes in retrovirus-induced animal tumors, approximately 25 years ago.[164] TKI's play an important role in the regulation of normal cellular processes and can be classified as being either receptor protein kinases or nonreceptor protein kinases. Receptor tyrosine kinases, which include over 60 identified receptors divided into 20 subfamilies, are membrane-spanning cell surface proteins that are involved in transducing extracellular signals to the cytoplasm.[165] Conversely, nonreceptor TKs relay intracellular signals and are located in the cytosol, nucleus, and the inner surface of the plasma membrane. Upon binding to a ligand, receptor TKs are autophosphorylated, leading to activation of multiple cytoplasmic signaling pathways, including: i) Ras/Raf mitogen-activated protein kinase pathway; ii) phophoinositol 3'-kinase/Akt pathway; iii) protein kinase C pathway; and iv) activation of scaffolding proteins.[166, 167] Within these pathways, intracellular mediators transduce signals from membrane receptors into the nucleus, resulting in altered DNA synthesis as well as maladaptation in cell growth, migration, differentiation, and death.[168, 169] Amongst the first TKIs developed with relative specificity against epidermal growth factor receptor (EGFR) were Imatinib, Gefitinib, and Erlotinib. Sunitinib, a protein TKI targeted against VEGFR-2, PDGFR, c-kit and Flt-3, was the first anti-cancer agent to be simultaneously approved for the treatment of two different indications.[170]

Sunitinib Anti-cancer Mechanisms

The development of TKIs as pharmacological agents targeted against cancer cells originates from the increased understanding of the biological mechanisms responsible for tumor cell progression. Multiple strategies have been proposed for the therapeutic efficacy of TKIs in the cancer setting. TKIs are known to compete with ATP for the

ATP-binding site of the tyrosine kinase catalytic domain. This results in the prevention of the angiogenesis intracellular signaling pathway from occurring. Other TKIs exert their effects by blocking the dimerization of fused tyrosine kinases, also inhibiting activation. Additionally, inhibitors of tyrosine kinase receptors interrupt TK signaling through: i) neutralization of the ligand; ii) blocking of ligand binding; iii) internalization of the receptor; and iv) antibody-mediated cytotoxicity.[171] Furthermore, TKIs interrupt the VEGF pathway by binding to pathway receptors, including VEGFR 1-3 and PDGFR α and β, thus inhibiting angiogenesis and tumor cell proliferation.[172]

Angiogenesis occurs in both physiologic and pathophysiologic settings and is a complex process involving the remodeling of established vasculature.[173, 174] Several angiogenic factors that play a critical role in angiogenesis include the endothelium-specific growth factors, such as VEGF family, the angiopoietin, and the ephrin families, as well as cytokines, proteinases, adhesion and junctional molecules.[175] VEGF, also known as vascular permeability factor (VPF), is a member of the PDGF family and demonstrates a high specificity for vascular endothelium. Regulation of vascular proliferation and permeability is achieved through the actions of VEGF, as well as cell migration and tube formation in order to support the formation of new vasculature.[67, 176, 177] In hypoxic conditions, most solid tumors and tumor-associated stroma secrete VEGF, which is found to be elevated in the setting of RCC. Although regulated by several growth factors and oncogenes [Mitogen-activated protein kinases (MAPKs) and P13K],[178] augmented VEGF expression has been discovered to correlate with tumor vascularity, cancer recurrence, and decreased survival rate.[179, 180] Endothelial cells of tumor vessels display specific surface receptors including, VEGF receptor-1 (VEGFR-1)

and VEGF receptor-2 (VEGFR-2). Circulating VEGF-A binds to both VEGFR-1 and VEGFR-2, which promotes endothelial cell migration and proliferation and results in the activation of intracellular TK, leading to the subsequent triggering of the intracellular signaling cascade.[181] Therefore, there is strong rationale to support the use of targeted agents for the inhibition of VEGFR TKs in the presence of RCC.

Sunitinib, also known as Sutent (SI11248), is a broad-spectrum, orally active TKI agent which targets multiple receptor kinases, including VEGFR 1-3, PDGFR α and β, KIT, FLT3, CSF1R, and rearranged during transfection (RET) receptor kinases.[182] (Figure 4). Due to the less selective design of Sunitinib, results of its therapeutic actions involve the inhibition of several different kinases. In a preclinical model, Sunitinib demonstrated selective *in vivo* inhibition of VEGFR-2 and PDGF- β phosphorylation, *in vitro* inhibition of endothelial cell and fibroblast proliferation, and anti-tumor effects in xenografts. Sunitinib was first approved by the FDA in January 2006 for the treatment of RCC and as well as gastrointestinal stromal tumor (GIST) in the setting of disease progression or development of imatinib mesylate intolerance. Presently, the use of Sunitinib is also indicated for the treatment of meningioma, pancreatic neuroendocrine tumors, metastatic breast cancer, and non-small cell lung cancer.[183]

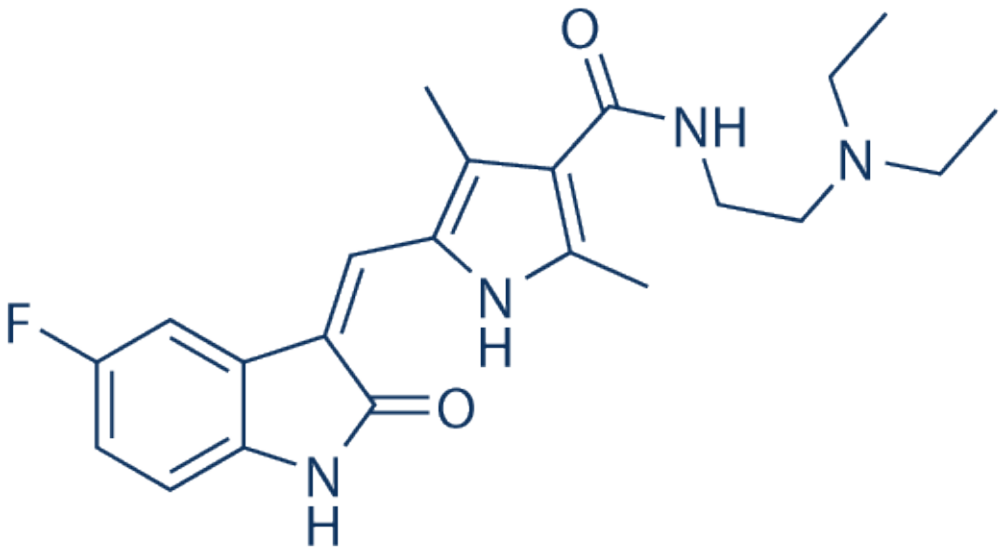


Figure 4: Chemical structure of Sunitinib (SU11248). *N*-[2-(Diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide, Malate Salt.

Sunitinib-induced Cardiotoxicity

Sunitinib is a novel multi-target therapy that is approved for the treatment of metastatic RCC and advanced GIST. The use of Sunitinib is also recommended as a second-line therapy in patients with drug intolerance and in the presence of malignant mutations in KIT (CD117), resulting in imatinib resistance.[184, 185] The recommended oral dosage for Sunitinib is 50 mg daily for 4 weeks, followed by a 2 week break from treatment, for a total cycle duration of 6 weeks.[186] Previously, cytokine-based therapy, including IL-2 and IFN-α, has demonstrated only low to medium response rates (6-20%) and had a substantial toxic burden.[187, 188] In comparison, Sunitinib for the treatment of advanced tumor progression has proven superior through the demonstration of increased treatment response rate and progression-free survival.[189] For this reason, Sunitinib is considered as the new standard in first-line treatment of metastatic RCC in this patient population. However, a deleterious downfall to the efficacy of Sunitinib is the fact that it targets multiple receptors,[190] potentially posing a greater risk of cardiotoxicity than more selectively targeted agents, such as imatinib. When assessed for the binding affinity to 119 tyrosine and serine/threonine kinases, Sunitinib demonstrated inhibition of numerous (>70 kinases) off-target kinases.[191] The broad spectrum actions of Sunitinib are likely responsible for the variety of adverse side effects observed clinically, among the most common include fatigue, diarrhea, hand-foot syndrome, stomatitis, hypothyroidism, myelotoxicity, and hypertension.[189, 192] Most concerning is the ability of Sunitinib to induce cardiac toxicity leading to potentially fatal cardiovascular complications in the cancer setting. Through the disruption of the VEGFR signaling cascade, capillary density is reduced and contractile dysfunction ensues, resulting in the development of fibrosis

and heart failure.[193, 194] Throughout the literature, various studies have demonstrated that in both animal and clinical models, the use of Sunitinib results in the development of hypertension, LV systolic dysfunction, and subsequent HF.[189, 195-198] (Figure 5)

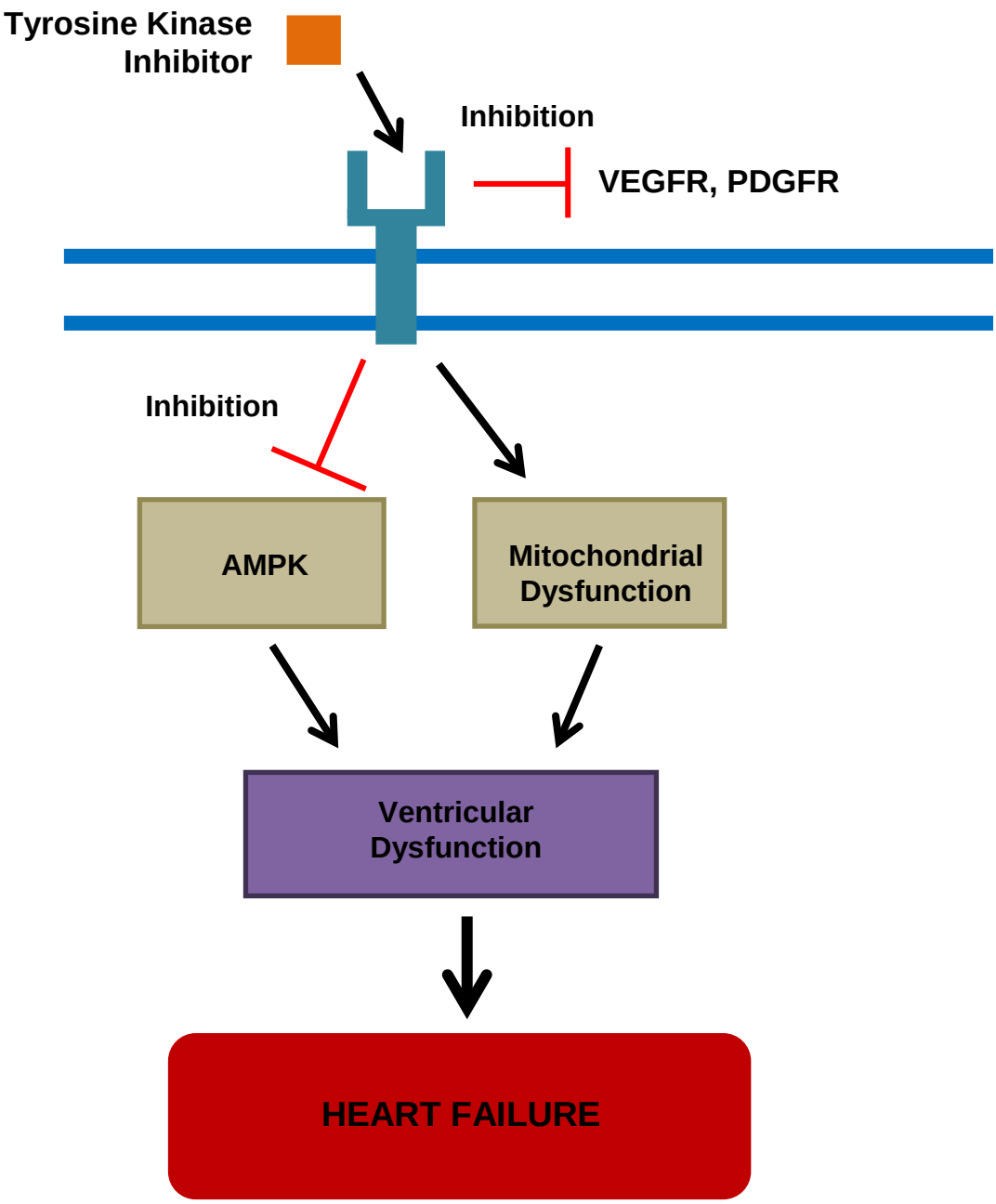


Figure 5: Potential mechanisms of Sunitinib induced cardiac dysfunction. Sunitinib targets VEGFR, PDGFR, and AMPK, resulting in ventricular dysfunction and heart failure. Vascular endothelial growth factor receptor, VEGFR; Platelet-derived growth factor receptor, PDGFR; AMP-protein activated kinase, AMPK.

i) Basic Science Model

The adverse cardiovascular outcomes that accompany RTK inhibition have been well documented in several FDA pharmacology reviews. In a pre-clinical model, Sunitinib potently inhibited the cardiac ion channel, human ether-a`-go-go- related gene (hERG), resulting in increased action potential duration in canine purkinje fibers.[199] The administration of Sunitinib at a clinically equivalent dosage results in QT interval prolongation and reduction in HR, as seen in an primate model. Upon histological analysis, mice exhibit evidence of drug-mediated pathology, including capillary proliferation, myocardial vascuolization, and pericardial inflammation.[199] Several potential mechanisms that are responsible for this drug-induced cardiotoxicity include: i) impairment of myocardial angiogenesis; ii) activation of oxidative stress (OS); and iii) inhibition of AMP-protein activated kinase (AMPK)[195, 196] (Figure 3). Apoptosis occurs through inhibition of AMPK signaling, which cause the JNK and p38 pathways to be activated, resulting in the enhanced expression of several pro-apoptotic genes, such as Bax, caspases, and poly ADP ribose polymerase (PARP).[200-202] A study conducted by Chu *et al*. which evaluated the effects of Sunitinib in animal cardiomyocytes, demonstrated increased mitochondrial swelling and disrupted cristae with transmission electron microscopy.[197] Sunitinib exposure was also observed to induce collapse of the mitochondrial membrane potential, leading to a decrease in intracellular ATP.[203] In an *in vitro* model, rat neonatal cardiomyocytes incubated with high-dose Sunitinib exhibited mitochondrial cytochrome-C release and activation of caspase-9, resulting in the initiation of the mitochondrial apoptotic pathway.[203] Under conditions of cardiac stress, AMPK also plays a critical role in detecting fluctuations in the adenosine monophosphate

(AMP):ATP ratio. By this ability, AMPK acts as a "master metabolic switch", inhibiting anabolic pathways and promoting energy generation in order to maintain homeostasis.[204] In an AMPKα2 knockout model, Zhang and colleagues revealed that following transverse aortic constriction (TAC), mice displayed greater loss of LV function, increased LV hypertrophy, and substantially higher mortality when compared to wild-type animals.[205] Therefore, recent studies suggest that impaired AMPK signaling may result in failed adaptations to systolic pressure overload and severe cardiac dysfunction.

Another potential explanation for the cardiotoxic side effects seen in Sunitinib treated animals is the inhibition of two critical growth factors, PDGF and VEGF. In the oncologic setting of RCC, Sunitinib acts to inhibit PDGR receptors α and β, leading to adverse effects in cardiac function. In a study by Edelberg *et al*., PDGR was discovered to mediate signaling between cardiac myocytes and adjacent endothelial cells.[206] Similarly, in a model of cardiac-specific PDGFR- β deletion performed by Chintalgattu and colleagues, animals subjected to TAC showed greater LV dilatation, reduced cardiac function, and increased pulmonary congestion.[207] Furthermore, inhibition of VEGFR 1-3 signaling pathways has proven detrimental to the regulation and maintenance of cardiovascular function.[208, 209] Administration of soluble fms-like tyrosine kinase receptor-1 (sFlt-1) (circulating form of VEGFR-1 that inhibits VEGF signaling) results in endothelial dysfunction and hypertension, which occurs through the antagonism and sequestration of VEGF and PIGF.[210, 211] Finally, delivery of a decoy VEGF receptor in an animal model of trans-aortic constriction is associated with decreased capillary density, enlargement of LV cavity dimensions, and decreased cardiac function.[212]

ii) Clinical Setting

Sunitinib serves as the standard of care for the first line treatment of advanced RCC, as well as in second line treatment of GIST patients resistant to imatininb. In addition to Sunitinib, several other TKIs have been approved by the FDA in the targeting of the VEGF signaling pathway, including pazopanib, sorafenib, and vandetanib. Various clinical trials have demonstrated the safety and efficacy of Sunitinib in the setting of cytokine refractory metastatic RCC,[213, 214] which ultimately lead to its approval in 2006. In the clinical evaluation of Sunitinib versus IFN-α, progression free survival was significantly greater in Sunitinib treated patients (11 months vs. 5 months, respectively $p<0.001$), corresponding to increased response rates (Sunitinib 31% vs. IFN-α 6%, $p<0.001$).[189] Additionally, a phase 3, randomized clinical trial demonstrated increased time to tumor progression in GIST patients treated with Sunitinib as compared to placebo (27.3 weeks vs. 6.4 weeks, $p<0.0001$).[198] Despite the clinical efficacy of Sunitinib in the management of progressive tumors, its use has become associated with a 25-30% risk of developing cardiotoxicity.[197, 215, 216] The main hypothesis surrounding the prevalence of Sunitinib-induced cardiotoxicity involves a dual mechanism. Acutely, inhibition of VEGF signaling results in a decrease in nitric oxide synthase (NOS) activity and reduced NO production,[105] which is responsible for the rapid changes in blood pressure observed in patients following treatment initiation.[217] Moreover, alterations in the PDGFR pathway lead to a reduction in cardiac capillary density and local tissue hypoxia.[207] Therefore, the development of hypertension incurs an afterload stress on the heart, which under normal conditions, is corrected for via compensatory mechanisms. However, inhibition of the PDGFR pathway prevents cardiac adaptation to afterload stress, and thus heart failure

develops. Finally, Sunitinib is known to interact with upwards of 50 kinases, which is more than any other FDA approved TKI agent.[218] Due to Sunitinib's reduced specificity, cardiotoxicity results as a consequence to the inhibition of multiple signaling pathways that may potentially play a critical role in the maintenance of ventricular function.

A variety of clinical investigations have demonstrated that hypertension is the most common cardiovascular toxicity among patients treated with Sunitinib.[219, 220] Severity of hypertension can be classified using the Common Terminology Criteria for Adverse Events (CTCAE) grading system, which was developed by the National Cancer Institute (NCI). Grades 1-4 hypertension can be categorized as follows: i) Grade 1- a transient (<24 hour), asymptomatic increase in diastolic blood pressure >20 mm Hg or to >150/100 mm Hg if prior normal limits; ii) grade 2- consists of recurrent or persistent (>24 hour) or symptomatic increase by >20 mm Hg or to >150/100 mm Hg if previously within normal limits; iii) grade 3- involves hypertension requiring more than one drug or greater intensive therapy; and iv) grade 4- is a hypertensive crisis. A meta-analysis evaluating 13 clinical trials and comprising 5000 patients found the incidence of all grade hypertension to be 22%, as well as a 7% incidence of grade 3-4 hypertension requiring adjustment to dosing of more than one medication.[219] A population of metastatic RCC patients treated with Sunitinib demonstrated systolic blood pressure greater than 140 mm Hg (81%) and diastolic blood pressure greater than 90 mm Hg (67% of patients).[220] A review performed by Hall and colleagues demonstrated that 32% of patients treated with Sunitinib developed hypertension and 30% developed heart failure, confirming the cardiotoxic side effects associated with TKIs.[221] In the setting of imatinib-resistant GIST, 47% of patients developed hypertension and 8% developed CHF after treatment with

Sunitinib.[197] Additionally, cardiac events have been seen to occur in approximately 1/3 of the clinical population, which are defined by the presence of: i) increased cardiac enzymes; ii) symptomatic arrhythmia requiring treatment; iii) newly developed LV dysfunction; and/or iv) acute coronary syndrome (ACS).[222] In a retrospective clinical study involving 48 RCC patients receiving Sunitinib, 15% developed symptomatic grade 3/4 LV systolic dysfunction within 3 months of treatment initiation.[216] Significant risk factors, which contributed to the development of LV systolic dysfunction, included low body mass index (BMI) and prior history of HF and/or CAD. Furthermore, a multicenter, randomized, phase 3 trial evaluated the use of Sunitinib against interferon alpha in 750 patients with previously untreated metastatic RCC. The incidence of LVEF to below normal values was observed in 10% of Sunitinib treated patients, with 2% of patients experiencing grade 3 LVEF impairment, as compared to 1% for the interferon alpha group.[189] Due to the recent knowledge that a decrease in LVEF >10% from baseline is associated with an overall reduction in 5 year survival,[223] early detection of cardiac dysfunction is important for the prevention of irreversible cardiac damage in this cancer population.

Early Detection of Drug-induced Cardiotoxicity

The use of novel targeted agents such as Bevacizumab and Sunitinib for the treatment of colorectal cancer and RCC, respectively, has demonstrated improved response rates and increased progression free survival when compared to chemotherapy alone.[83, 97] However, a limiting aspect of their use is an increased risk of developing cardiotoxicity, including, hypertension, reduced LVEF, and heart failure.[81, 100, 216] Since the introduction of chemotherapeutic agents in the early 1940's, various surveillance

modalities, such as radionuclide angiocardiography, multiple-gated acquisition scanning, and echocardiography have been implemented for the detection of myocardial dysfunction. Throughout the past 50 years, serial assessment of LVEF has served as the most clinically relevant and validated method for monitoring cardiotoxicity in the cancer population.[224-226] According to the recent guidelines set by the Cardiac Review and Evaluation Committee of Trastuzumab-associated cardiotoxicity, the definition of cardiotoxicity involves the following: i) a reduction of LVEF of ≥5% to <55% with symptoms of heart failure; or ii) an asymptomatic reduction of LVEF of ≥10% to <55%.[227] In association with reduced LVEF, the development of asymptomatic or symptomatic heart failure may be indicative of irreversible cardiac injury.[228] Therefore, in order to detect early-stage cardiac dysfunction prior to a decrease in LVEF, the use of non-invasive techniques would be clinically useful for the prevention and treatment of drug induced cardiotoxicity.

i) Cardiac Biomarkers

Within the last decade, the novel use of cardiac biomarkers including troponins, C-reactive protein (CRP), and brain natriuretic peptide (BNP) has emerged as a reliable diagnostic tool for the early recognition of drug-mediated cardiotoxicity. The clinical assessment of biomarkers serves as a superior monitoring approach for a variety of reasons, such as: i) the procedure is minimally invasive; ii) it is associated with reduced cost compared to echocardiography or nuclear techniques; iii) there is no harm of radiation exposure to the patient; and iv) interpretation of the results does not require the expertise of a specialist.[229] Serum levels of cardiac troponins, specifically Troponin-T (TnT) and Troponin-I (TnI), are considered to be the most sensitive biomarkers for

assessing myocardial damage and in the diagnosis of acute myocardial infarction.[230] Clinically, elevated troponin blood levels may be used for cardiac risk stratification among patients, and are known as reliable biomarkers for a broad range of cardiac pathologies including, left ventricular hypertrophy, congestive heart failure, pulmonary embolism, and anti-cancer drug mediated cardiotoxicity.[231] TnT and TnI consist of three different isoforms with unique structures, including one for slow-twitch skeletal muscle, one for fast-twitch skeletal muscle, and one for cardiac muscle.[232, 233] Mechanisms such as ventricular remodeling, neurohormonal effects on the myocyte, presence of coronary artery disease in congestive heart failure, and coronary microcirculation abnormalities result in the release of TnT and TnI into the circulation.[234] Additionally, natriuretic peptides such as n-terminal pro-brain natriuretic peptide, brain natriuretic peptide, and atrial natriuretic peptide, have been identified as predictive markers for severity of heart failure.[235] In the presence of increased ventricular wall stress, these biomarkers are released into the serum by injured cardiomyocytes. Recently, various markers of endothelial dysfunction [plasminogen activator inhibitor (PAI-1) and tissue type plasminogen activator (t-PA)],[236] MI [fatty acid binding protein (FABP) and glycogen phosphorylase isoenzyme BB (GPBB)],[237] and inflammation (IL-6 and TNF-α)[238] have also been studied in the Cardio-Oncology setting; however their utility in predicting cardiotoxicity remains to be determined.

Numerous clinical studies have evaluated the significance of troponin levels following the administration of high-dose chemotherapy. In the breast cancer setting, TnI was shown to be a sensitive and specific marker for myocardial injury in chemotherapy-treated patients, and served as an early predictor of left ventricular dysfunction

development.[239, 240] A prospective study evaluating 703 patients receiving high dose chemotherapy demonstrated that the absence of TnI elevation following chemotherapy was associated with normal LVEF and low incidence of cardiac events (1%) throughout a three year-long follow-up.[241] Auner and colleagues demonstrated that in patients treated with standard dose anthracyclines, 15% showed an increase in TnT, which corresponded to a significant reduction in LVEF when followed over a 12 month period.[242] Furthermore, in TnI-positive patients suffering from cardiac dysfunction, there is 3-fold greater chance of no recovery following heart failure therapy, as compared to TnI-negative patients (relative risk, 2.9; CI 95%, 1.7-3.9; $P < 0.001$).[243] In addition, several studies have confirmed that the administration of anticancer drugs, specifically anthracyclines, induce subclinical myocardial injury, which is characterized by increasing levels of BNP.[244, 245] The evaluation of various cardiac biomarkers including TnT, CRP, and BNP has recently been analyzed in the setting of anthracycline (DOX) and monocloncal antibody (TRZ) combination therapy.[228] In a population of HER-2 positive breast cancer patients treated with adjuvant DOX+TRZ, the utility of cardiac biomarkers was evaluated for predicting early LV systolic dysfunction. A total of 10 (25%) women developed DOX+TRZ induced cardiotoxicity, in which neither TnT, CRP, or BNP were able to predict early LV systolic dysfunction. Despite these findings, a study by Cardinale *et al.* demonstrated that frequent sampling of TnI was able to identify a subset of women at high risk of DOX+TRZ mediated cardiac dysfunction, prior to a decrease in LVEF.[229] In addition, a recent study by Ky *et al*. evaluated the role of various biomarkers for the prediction of cardiotoxicity, in breast cancer patients treated with DOX, Taxanes, and TRZ.[246] Biomarkers including TnI and CRP demonstrated a significant increase from

baseline to 3 months ($p<0.05$), whereby patients with a greater change in biomarker level demonstrated a 47% risk of cardiotoxicity. The question remains whether these biomarkers, including high sensitivity troponins, can serve as early predictors of Bevacizumab and Sunitinib mediated cardiac dysfunction in this unique cancer population.

ii) Conventional Echocardiography

Non-invasive assessment of LVEF using multiple-gated acquisition (MUGA) scintigraphy and transthoracic echocardiography (TTE) are the most common methods of monitoring cardiac function in the cancer setting.[228, 247] MUGA is a reliable and widely accepted method for the serial assessment of LVEF and has been shown to be predictive of heart failure development.[248] However this technique involves a substantial amount of radiation, equivalent to approximately 100 chest x-rays, and may inaccurately assess LVEF in patients with underlying arrhythmias.[225] Due to it's low cost, portability, and lack of radiation, conventional TTE is more commonly used in the clinical setting for the measurement of LVEF and fractional shortening (FS).[249] Despite the frequent use of LVEF as a measure of cardiac function, compensatory reserve of the myocardium enables adequate ventricular output in the presence of dysfunctional myocytes, and thus, the extent of cardiac injury is often undervalued.[250] A variety of studies have demonstrated the inability of LVEF measurement to detect LV dysfunction, as normal LV systolic function is often persistent despite underlying diastolic impairment. Swain and colleagues analyzed several phase III clinical trials, comprising a total of 630 patients treated with Doxorubicin.[3] During treatment, 149 patients were determined to have a decrease in LVEF (>30% from baseline) and 32 patients developed chronic heart failure.

However, only 11/32 patients who developed chronic heart failure demonstrated a preceding decrease in LVEF, suggesting that early changes in LVEF are unable to predict anthracycline-induced heart failure. In a prospective study of 120 breast cancer patients, LVEF was measured up to 3 years after treatment with anthracycline.[251] Severe reductions in LVEF (decrease of >35% from baseline) were demonstrated in 12/120 patients, in which 11/12 occurred more than 3 months after treatment was stopped. Additionally, cardiac systolic function was shown to progressively deteriorate throughout the 3 year follow-up. Finally, LVEF measurement is shown to have a broad range of values in healthy individuals, as well as high intra- and inter-observer variation, which limits its reproducibility. Therefore, as conventional echocardiography evaluation of LVEF is unable to detect subtle physiological changes in the myocardium, the investigation of more sensitive imaging techniques is needed to accurately detect early cardiac dysfunction.

iii) Tissue Doppler Imaging: Endocardial Velocity and Strain Rate

Novel echocardiographic techniques including tissue Doppler imaging (TDI) and strain rate imaging (SRI) have been developed to improve the diagnostic value of non-invasive echocardiography.[249] Unlike conventional LVEF assessment, TDI measures are less influenced by loading conditions and allow for the evaluation of diastolic and systolic velocities of the ventricular walls and the mitral annulus.[252] Myocardial Doppler imaging involves the evaluation of parameters shown to be closely associated with cardiac function, including velocity, deformation (strain), and deformation rate (strain rate), and can be measured with both high quality spatial and temporal resolution.[253, 254] In a murine model, endocardial systolic velocity (V_{endo}) is evaluated in cm/s, whereby a

value of >3 cm/s is considered normal and a decline of V_{endo} below 2 cm/s is indicative of myocardial dysfunction.[255] A limitation of the measurement of V_{endo} with TDI includes the misinterpretation of translational motion of the image in relation to the transducer for tissue movement due to myocardial contraction. Fortunately, SRI, which measures the degree of myocardial compression and deformation, is performed in addition to TDI to overcome this constraint.[249] Whereas TDI evaluates one point on the myocardium in relation to the transducer, SRI compares two points on the myocardium in relation to each other. Strain rate (SR) is measured as a speed of deformation ($seconds^{-1}$) which, in an animal model, a value of $>20s^{-1}$ is considered within normal limits.[249] At a time when LVEF remains within normal limits, measurement of V_{endo} and SR allow for the reproducible assessment of systolic and diastolic function including the early detection of cardiac dysfunction.[255]

The recognition of TDI and SRI as being sensitive, non-invasive echocardiographic techniques that allow for the early detection of sub-clinical cardiac injury has been demonstrated in a myriad of studies. At the basic science level, Neilan *et. al* demonstrated that in doxorubicin-treated mice, TDI-derived parameters were able to detect LV dysfunction prior to alterations in conventional indices such as, heart rate, LV end-diastolic pressure, and conventional echocardiography.[256] More importantly, these novel parameters were able to predict the development of late cardiac dysfunction and mortality after treatment with doxorubicin. A study by Jassal *et al*. evaluated the role of TDI as an early sensitive marker of LV systolic dysfunction in mice treated with DOX, TRZ, or a combination of both agents.[255] In DOX+TRZ treated animals TDI results were shown to be abnormal as early as 24 hours after administration and were predictive of

ensuing LV systolic dysfunction and reduced survival. Translating these basic science findings to the clinical arena, Tassan-Mangina *et al*. evaluated the early and late effects of cumulative DOX treatment using conventional echocardiography and TDI.[257] Results demonstrated alterations in diastolic function including reduced isovolumic relaxation time, which served as an accurate predictor of reduced LVEF below 50%. Additionally, Fallah-Rad and colleagues recently evaluated the role of TDI in the early detection of DOX+TRZ mediated cardiac dysfunction in women with breast cancer.[228] Of the 42 patients prospectively followed between 2007-2010, 10 (25%) women developed DOX+TRZ mediated cardiomyopathy. Both TDI and SRI were able to detect pre-clinical changes in LV systolic function as early as 3 months, prior to conventional changes in LVEF at 6 months. The novel discovery of TDI as an early sensitive marker of LV systolic dysfunction in the breast cancer setting has recently been confirmed by five independent studies.[93, 258-260] Early detection of DOX+TRZ mediated cardio-toxicity with TDI could potentially allow for the adjustment of treatment and/or prophylactic administration of cardio-protective agents in women with breast cancer, prior to the development of irreversible cardiac dysfunction.[255] Whether these novel TDI techniques can be applied for the early detection of BVZ or SNT mediated cardio-toxicity requires further study.

Chapter 2: Materials and Methods

Study Rationale

Working Hypothesis

Cardiovascular toxicity is a severe short and long-term complication of anti-cancer therapy with Bevacizumab and Sunitinib. The early detection of Bevacizumab and Sunitinib mediated cardiotoxicity using cardiac biomarkers and novel imaging techniques, may enable the adjustment of treatment with the anti-cancer drug and/or the administration of cardioprotective agents, prior to the development of irreversible cardiac dysfunction.

Aim: To evaluate whether cardiac biomarkers and/or echocardiographic techniques, specifically TDI and SRI, can noninvasively detect the early manifestations of cardiac dysfunction prior to a decrease in LVEF, in mice treated with either Bevacizumab or Sunitinib.

Hypothesis: We hypothesize that cardiac biomarkers, TVI, and/or SR imaging will be able to accurately detect subtle cardiac injury at a time when LVEF remains normal, in a murine model of Bevacizumab or Sunitinib mediated cardiotoxicity.

Animal Model

All animal procedures were conducted in accordance with guidelines published by the Canadian Council on Animal Care. All procedures, including drug administration and longitudinal echocardiographic studies, were approved by the Animal Protocol Review Committee at the University of Manitoba. A total of 95, wild-type C57Bl/6 (8-10 weeks old), male mice were randomized into four groups receiving the following drug regimens (Figure 6):

1. 0.9% Saline (i.p., n=5)
2. Doxorubicin (20 mg/kg, i.p., n=20)[255]
3. Bevacizumab (10 mg/kg, i.v., n=35)[261]
4. Sunitinib (40 mg/kg/day, orally, n=35)[197]

All mice were quarantined for 1 week, prior to randomization. At baseline, all mice underwent baseline transthoracic echocardiography (TTE) and weight analysis. Single intraperitoneal (IP) injections of 0.9% saline or DOX (20 mg/kg), as well as intravenous (IV) injection of BVZ (10 mg/kg) were administered following baseline data acquisition (Figure 7). Animals treated with DOX served as positive controls throughout the study. Sunitinib (40 mg/kg/d) was administered via daily oral gavage for a total of 14 days (Figure 7). Our group has extensive experience in establishing both acute and chronic murine models of chemotherapy induced cardiac dysfunction. Serial echocardiography and weight measurements were performed daily for 14 days, at which time all surviving mice were anesthetized using pentobarbital (110 mg/kg, i.p.) and hearts were preserved for histological and biochemical analyses. (Figure 7). The above doses of anti-cancer drugs were used as these are the minimum concentrations required to induce LV systolic dysfunction in a murine model.

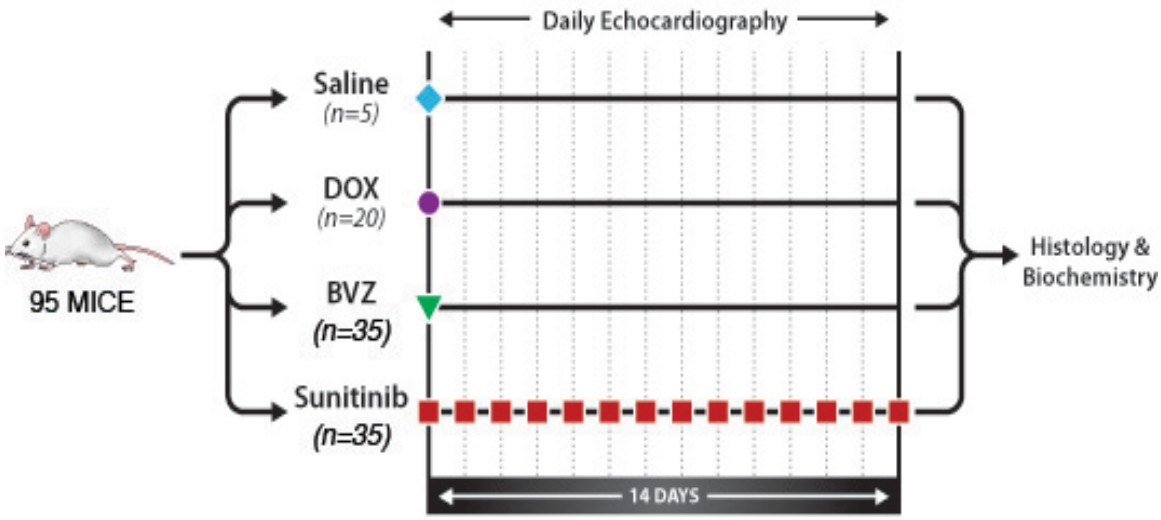


Figure 6: Animal protocol Timeline

In an acute murine model of chemotherapy induced cardiac dysfunction, a total of 95 wild-type C57Bl/6 male mice were randomly assigned to one of the following drug regimens: i) 0.9% saline (i.p., n=5); ii) DOX (20 mg/kg i.p., n=20); iii) BVZ (10 mg/kg i.v., n=35); or iv) SNT (40 mg/kg/d orally, n=35). Both DOX and BVZ were administered once during the experimental protocol via parenteral injection. Sunitinib was administered via oral gavage for a total of 14 days. *In vivo* cardiac function using TDI was assessed daily in surviving animals and high sensitivity TnI (hsTnI) was measured at baseline, day 7, and day 14. At baseline and day 14, hsTnI was measured to determine extent of cardiotoxicity from either BVZ or SNT. The early time point of day 7 was selected to detect early cardiomyopathy. At the end of the experiment, the mice were sacrificed for determination of histological and biochemical analyses. DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib.

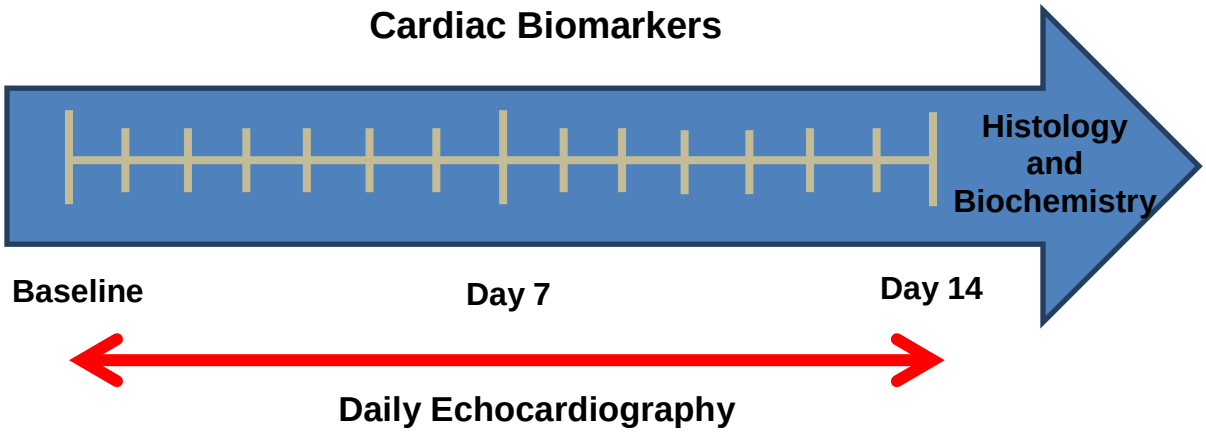


Figure 7: Timeline of Analyses

Prior to receiving acute treatment (0.9% saline, DOX, BVZ, or SNT), all mice underwent baseline echocardiography to determine cardiac function. Following initiation of therapy, mice were followed daily to assess cardiac dimensions and function. At day 14, all surviving mice were euthanized and hearts removed for histological and biochemical analyses. DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib.

Echocardiography

Non-invasive assessment of cardiac function was performed via murine echocardiography in awake mice beginning at baseline and daily for the next 14 days.[255] Echocardiographic data was collected using a 13-MHz probe (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) capable of TVI acquisition. All mice were imaged in the parasternal long axis (PLAX) windows, as previously described.[247, 262, 263] Post-processing of all images was conducted offline using the EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US). Images acquired in the PLAX window were collected for the calculation of LVEF, which was determined by manually tracing the LV end-diastolic and end-systolic volumes (Equation 1). M-mode echocardiography was also conducted for the evaluation of LV cavity dimensions including, LV end-diastolic diameter ($LVID_{ED}$), LV end-systolic diameter ($LVID_{ES}$), posterior wall thickness (PWT) and interventricular septal thickness (IVS).[247, 264] The GE EchoPAC PC program, version 11.2 was also used in the calculation of fractional shortening (FS) and LVEF (Equations 1 and 2).

Equation 1: Left Ventricular Ejection Fraction

$$\text{LVEF} = \frac{LV\ end\ diastolic\ volume - LV\ end\ systolic\ volume}{LV\ end\ diastolic\ volume}\ x\ 100\%$$

Equation 2: Fractional Shortening

$$\text{FS} = \frac{(LVEDD - LVESD)}{LVEDD}\ x\ 100\%$$

Tissue velocity imaging was acquired in the parasternal short axis (SAX) window at the level of the papillary muscles, at a rate of 483 frames per second.[264] A region of interest (0.2 x 0.2 mm) was manually positioned along the posterior wall of the endocardium for the measurement of peak endocardial velocity (V_{endo}) (Figure 8). Echopac PC software (GE Medical, Milwaukee, WI, US) was used to measure radial SR over an axial distance of 1 mm (width 0.6 mm). During analyses, the temporal smoothing filters were turned off for all measurements. An average value was calculated using 5 consecutive cardiac cycles.

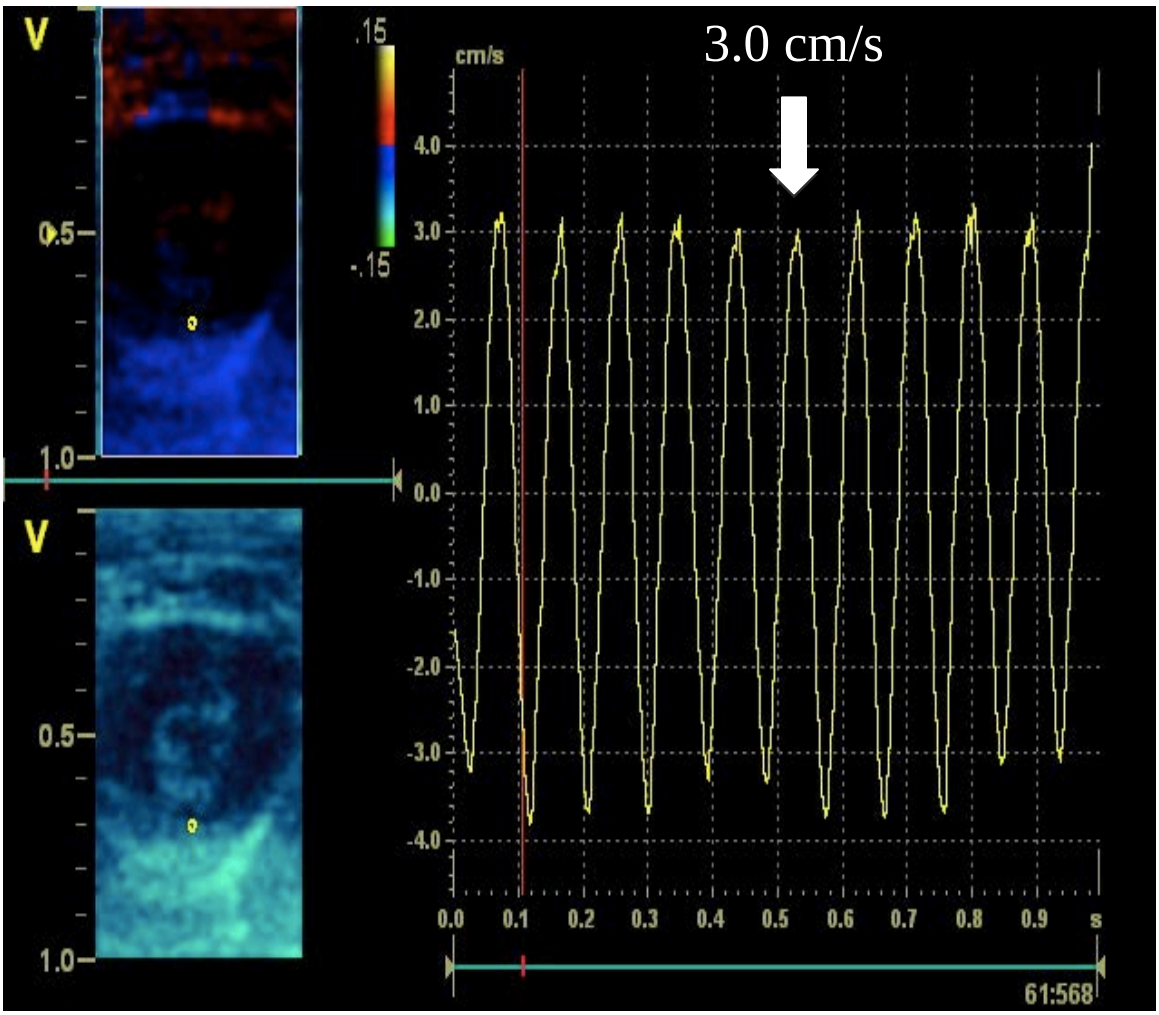


Figure 8: Tissue Velocity Imaging

Tissue velocity imaging of a normal healthy mouse. A V_{endo} for mice between 2.5 and 3.5 cm/s is considered to be within the normal range. A V_{endo} of less than 1 cm/s is indicative of cardiac systolic dysfunction. V_{endo}, peak endocardial systolic velocity.

Hemodynamics

Non-invasive measurement of heart rate (HR) and blood pressure (BP) was performed on conscious, restrained mice using a tail cuff method (CODA system, High Throughput, Kent Scientific, Torrington, CT), as previously described.[265] Briefly, the holding platform was heated to 30°C, at which time 5 BP readings were recorded with 1 minute rest intervals between readings. At baseline and day 14, BP measurements were collected, from which average values for systolic and diastolic BP were calculated using the 5 individual readings.

Cardiac biomarkers

In all surviving mice, approximately 100 μL of blood was collected via the jugular vein at baseline and day 7. Upon euthanization, the heart was removed and all blood remaining in the thoracic cavity was collected through pipette. All blood samples were placed on ice after collection and centrifuged at 1300g for 15 minutes in order to obtain the serum, which was stored at -80°C until biochemical analysis procedures were completed.

After the completion of the study, mouse-specific enzyme-linked immunosorbent assays (ELISAs) were performed on the collected serum to determine the presence of the cardiac biomarker TnI. A High Sensitivity Mouse Cardiac Troponin-I ELISA Kit (Life Diagnositics, Inc. Cat. No. 2010-1-HS) was used for all samples. Due to the low total volume collected, each sample was diluted 4x for analysis. Standard solutions of TnI were prepared by serial dilution. A total of 100 μL of each standard and diluted sample were dispensed in duplicate and distributed into predetermined wells. A volume of 100 μL TMB reagent (Life Diagnostics, Inc. Lot P- K2012A) was dispensed into each well

and mixed using an orbital shaker for 20 minutes. Stop solution (Life Diagnostics, Inc. Lot L- A0213A) was then added and mixed gently. Finally, absorbance at 450 nm was read with a plate reader (MRX Microplate Reader, Dynex Technologies Inc. 1CXD-4588, Chantilly, VA, US) and sample results were extrapolated from the generated curve.

Histology

At day 14, all surviving mice were euthanized and hearts were removed from the thoracic cavity. Each heart was rinsed and cleaned in 0.9% saline and halved. Half of the heart was collected for histological analysis via electron microscopy. The other half of the heart was flash frozen in liquid nitrogen for biochemical assays.

In preparation for electron microscopy, half hearts were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (ph 7.3) at room temperature for 3 hours. Tissues were rinsed over night at 4°C in 0.1 M phosphate buffer containing 5% sucrose. Tissues were then post-fixed at room temperature with 1% osmium tetroxide in .01M phosphate buffer for 2 hours. Samples were then dehydrated in ascending concentrations of ethanol and embedded in Epon 812 using standard techniques.[266] Thin sections were stained with uranyl acetate and lead citrate, viewed, and photographed on a Philips CM12 electron microscope to determine the extent of cell degradation. Grids were coded without prior knowledge of their source to eliminate observer bias. Wet weights of liver and lung samples were measured for all mice at the time of euthanization. Tissue samples were dried for 72 hours at 65°C and dry weights were used to determine wet-to-dry weight ratios.

Western Blotting

Frozen heart tissue was ground in liquid nitrogen and proteins were extracted in the radioimmunoprecipition (RIPA) buffer containing protease and phosphatase inhibitors (Thermo Scientific). Following a 20 min incubation period on ice, samples were centrifuged at 10, 000 rpm at 4°C for 10 minutes. Supernatants were then removed in pre-chilled labeled tubes and proteins were quantitated using the BioRad protein assay. Protein lysates were stored at -80°C until future analysis by Western blotting. For Western blotting, 30 μg of protein was loaded in each lane and samples were electrophoresed in a 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) at 100V for 1.5 h using the Mini-PROTEAN Tetra System (BioRAD) at room temperature. Separated proteins were transferred to a 0.45-mm PVDF membrane (Roche Diagnostics) for 2 hours at 100V at 4°C. Membranes were stained with Ponceau S to detect for the transferred proteins and stain was removed by washing with 1X TBST (Tris buffered saline with 0.1% Tween 20). Membranes were then blocked for 1 hour in 1X BSA blocking buffer/TBST (Thermo Scientific), which was reconstituted from the 10X stock. Membranes were incubated with primary antibodies overnight at 4°C in sealed pouches. The following day, membranes were washed 3X (10 min each wash) in 1X TBST and subjected to incubation with the secondary antibody for 45 min at room temperature. Membranes were washed 3X (10 min each wash) with 1X TBST. Proteins in the membrane were detected using the ECL Plus detection reagent (Western Lightning Plus-ECL, Amersham) and detected by X-ray film exposure. Primary antibodies used in this study were polyclonal antibodies to Bax, Caspase-3, and PARP (Cell Signalling) respectively. For the loading control, polyclonal antibody to GAPDH (Sigma) was used.

Stripping was achieved by incubating the membranes with Restore PLUS Western blot Stripping buffer (Thermo Scientific) for 15 min at room temperature, washing with 1X TBST (3X), reprobing with GAPDH (15 min), at which point the signals were detected as before. Band intensity was quantitated using the image analysis software (Quantity One; BioRad Laboratories, Inc).

Statistical Analysis

All data are expressed as mean±SD. Statistical significance between echocardiographic measurements was determined using a 1 (Genotype) x 2 (Time) mixed factorial design with repeated measures on the time factor. For post-hoc analysis, repeated measures of ANOVA were used to evaluate for significance between independent factors. In post-hoc between group analysis, Levene's test was used to check for homogeneity of group variances. P-values for main effects and interactions were also recorded where appropriate. Histological analyses involved non-parametric comparison of scores, ranging from 1-4 was calculated using the Kruskal-Wallis test, with 4 representing severe damage. A p value of <0.05 was considered significant. For biochemical and western analyses, a Student t-test was performed. A p-value of less than 0.05 was considered significant. The statistical analysis package SPSS 15.0 and Graphpad Prism 5 were used to perform the analysis.

Chapter 3: Results

Survival

Throughout the course of the study, there was no mortality observed in mice treated with BVZ or SNT by day 14 (end of the study). There was an 80% survival rate at day 3, in DOX-treated mice, with survival progressively declining beginning at day 4 until day 7. By day 8, there was 100% mortality in mice receiving DOX, which served as a positive control during the course of the study.[255] See Figure 9 for percent survival data of all groups of mice during the 14 day trial.

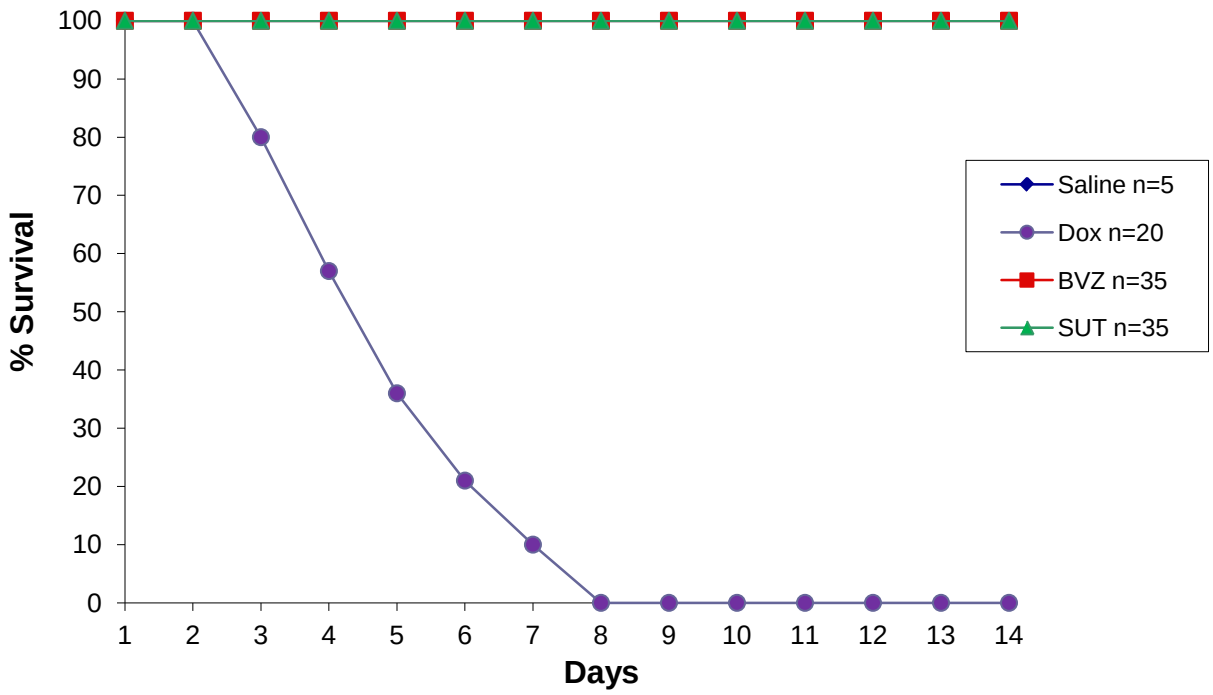
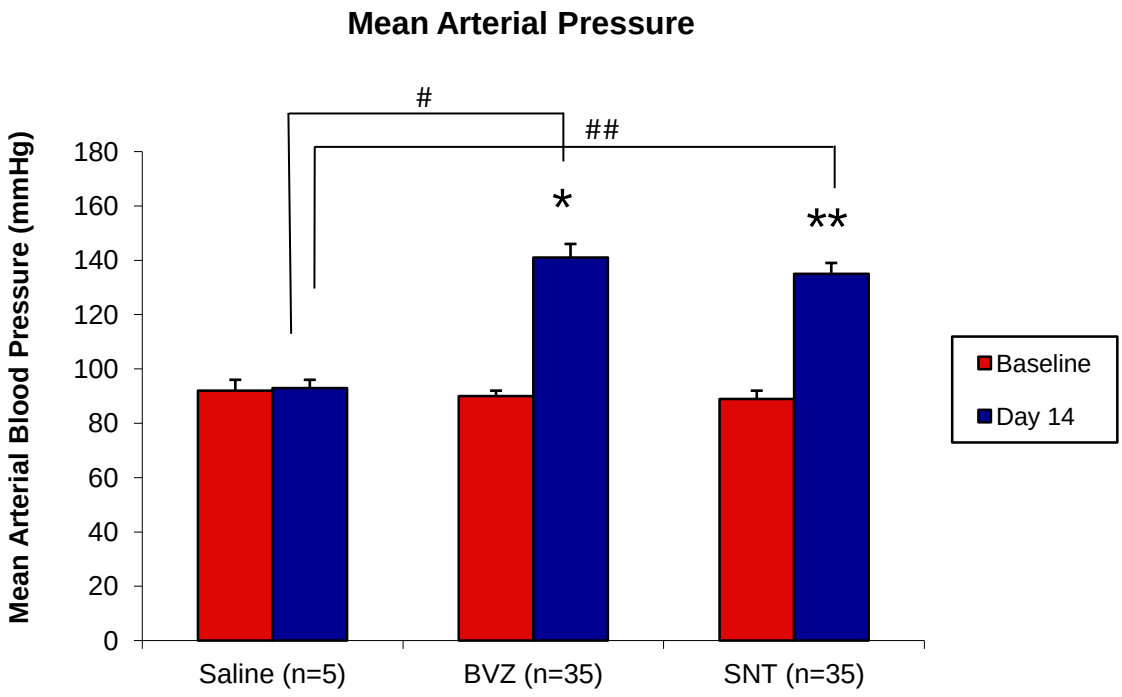


Figure 9: Percent survival of different treatment groups. There was no mortality in mice treated with saline, BVZ, and SNT up until day 14 when animals were euthanized. There was 100% mortality in DOX treated mice observed by day 8 of the 14 day study. DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib.

Hemodynamics

Baseline measurements of systolic and diastolic blood pressures were within normal limits for all four treatment groups. At day 14, mean arterial blood pressure (MAP) of saline mice remained unchanged as compared to baseline. Mice treated with BVZ showed a significant increase in MAP from 90±2 mmHg to 141±5 mmHg, $p<0.05$ (Figure 10). Similarly, mice treated with SNT showed a significant increase in MAP from 89±3 mmHg at baseline to 135±4 mmHg at day 14, $p<0.05$. As the mice treated with DOX demonstrated 100% mortality by day 8, we were unable to obtain day 14 blood pressure results for DOX-treated mice. See Figure 10 for blood pressure values measured at baseline and day 14.



*p<0.05 between BVZ at day 14 as compared to baseline
**p<0.05 between SNT at day 14 as compared to baseline
#p<0.05 between BVZ and saline at day 14
##p<0.05 between SNT and saline at day 14

Figure 10: Mean arterial blood pressures at baseline and day 14 in mice treated with BVZ and SNT, in comparison to mean arterial blood pressure of saline controls. No hemodynamic data for DOX animals as mortality rate was 100% at day 8. DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib.

Echocardiography

At baseline, heart rate, LV cavity dimensions, and systolic function were similar between all treatment groups. Heart rate (HR) and PWT remained within normal limits throughout the duration of the 14 day study for all treatment groups, except for the DOX group (Table 1). In DOX treated mice, the LV cavity dilated over time from 3.2±0.2mm to 3.6±0.2mm at day 5. In addition, treatment with DOX caused a significant decrease in the tissue Doppler indices V_{endo} from 3.2±0.2cm/s to 2.7±0.1cm/s and SR from 21±2s^{-1} to 18±1s^{-1} as early as 24 hours after treatment. These results verify DOX as being a positive control for the early detection of drug induced cardiotoxicity.

In both the BVZ and SNT treated mice, conventional echocardiographic indices showed a significant increase in LVEDD and decrease in LVEF beginning at day 13 (Figure 11). At day 13, in the BVZ treatment group, LVEDD initially increased from 3.1±0.2mm at baseline to 3.9±0.2mm at day 14. In the SNT treatment arm, LVEDD increased from 3.1±0.2mm at baseline to 3.9±0.3mm at day 14 (Table 1). These findings confirm the development of LV cavity dilation in both treatment groups by the end of the study.

Tissue velocity parameters, including V_{endo} and SR, decreased at day 8 in both BVZ and SNT treatment groups (Figure 12 and 13). Both BVZ and SNT treatment groups demonstrated normal V_{endo} and SR values at baseline. In mice treated with BVZ, V_{endo} decreased from 3.5±0.3cm/s at baseline to 2.4±0.1cm/s at day 8, and continued to decrease to 1.3±0.1cm/s at day 14. Strain rate decreased from 21±1s^{-1} at baseline to 14±2s^{-1} at day 8 and continued to decrease to 9±1s^{-1} at day 14 (Figures 12 and 13). Similarly, in SNT treated mice, V_{endo} decreased from 3.4±0.2cm/s at baseline to

2.5±0.2cm/s at day 8, and continued to decrease to a final value of 1.2±0.2cm/s at day 14. In addition, SR decreased from 21±2s^{-1} at baseline to 15±2s^{-1} at day 8 and continued to decrease to 10±2s^{-1} at day 14 (Figure 12 and 13). A decrease in LVEF of approximately 30% at day 13 correlates with a 30% decrease in V_{endo} by day 8. Therefore, in animals treated with either BVZ or SNT, TVI parameters (V_{endo} and SR) were able to detect evidence of LV systolic dysfunction 5 days prior to conventional echocardiographic indices (LVEDD and LVEF).

Table 1: Echocardiographic data

Echocardiographic Variable	Group	Baseline	Day 14
HR (Beats/min)	Saline	623±9	630±14
	BVZ	615±14	642±14
	SNT	615±14	623±11
PWT (mm)	Saline	0.72±0.02	0.71±0.03
	BVZ	0.72±0.02	0.73±0.02
	SNT	0.73±0.02	0.74±0.02
LVEDD (mm)	Saline	3.1±0.2	3.1±0.2
	BVZ	3.1±0.2	3.9±0.2*
	SNT	3.1±0.2	3.9±0.3*
LVEF (%)	Saline	72±2	74±2
	BVZ	75±3	48±2*
	SNT	74±3	47±3*
V_{endo} (cm/s)	Saline	3.4±0.2	3.1±0.2
	BVZ	3.5±0.3	1.3±0.1*
	SNT	3.4±0.2	1.2±0.2*
SR (s^{-1})	Saline	21±1	22±2
	BVZ	21±1	9±1*
	SNT	21±2	10±2*

Table 1: Echocardiographic data at baseline and day 14 (mean ± SEM) from mice receiving either 0.9% saline, BVZ, or SNT chemotherapy treatment. *$p<0.05$ as compared to baseline. DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib; HR, heart rate; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; V_{endo}, peak endocardial systolic velocity; SR, strain rate.

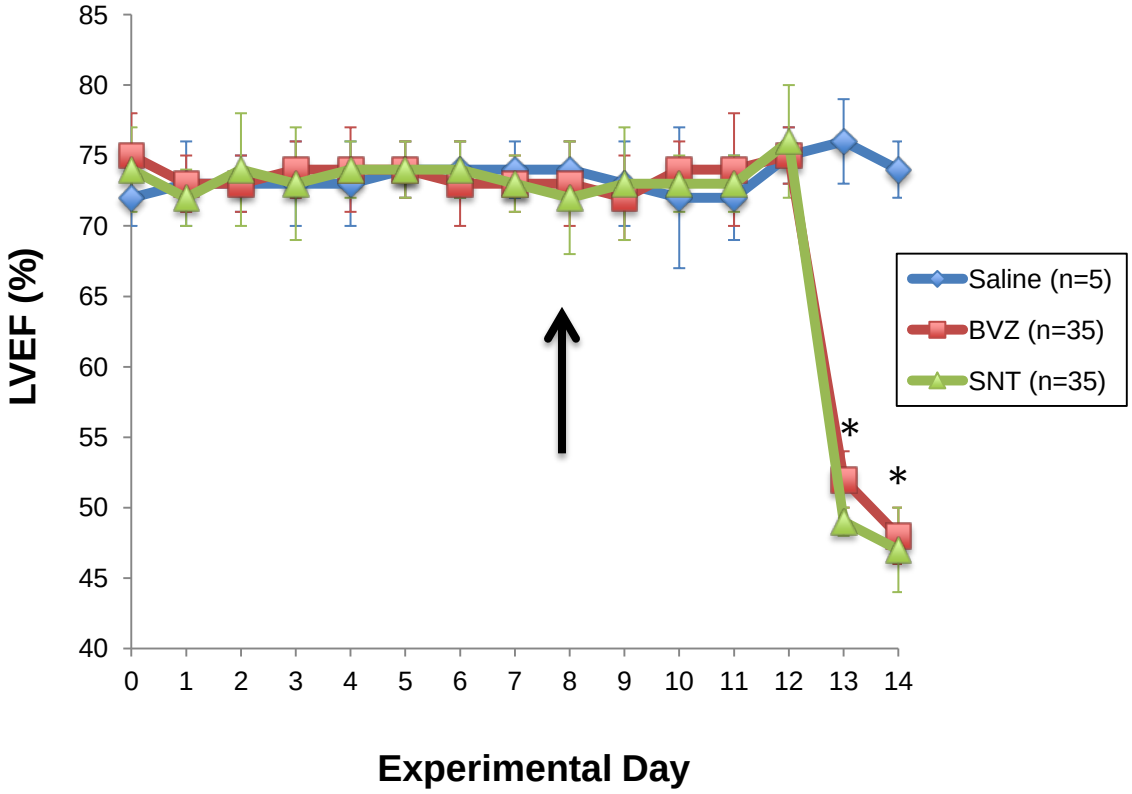


Figure 11: Left ventricular ejection fraction [LVEF (%)] of saline, BVZ-, and SNT-treated mice as determined by M-mode conventional echocardiography. LVEF decreased at day 13 in BVZ and SNT-treated mice. A decrease in LVEF of approximately 30% at day 13 correlates with a 30% decrease in V_{endo} by day 8. (Arrow indicates early time-point of decreased Vendo and SR at day 8). *$p<0.05$ as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; $V_{endo,}$ peak endocardial systolic velocity; SR, strain rate.

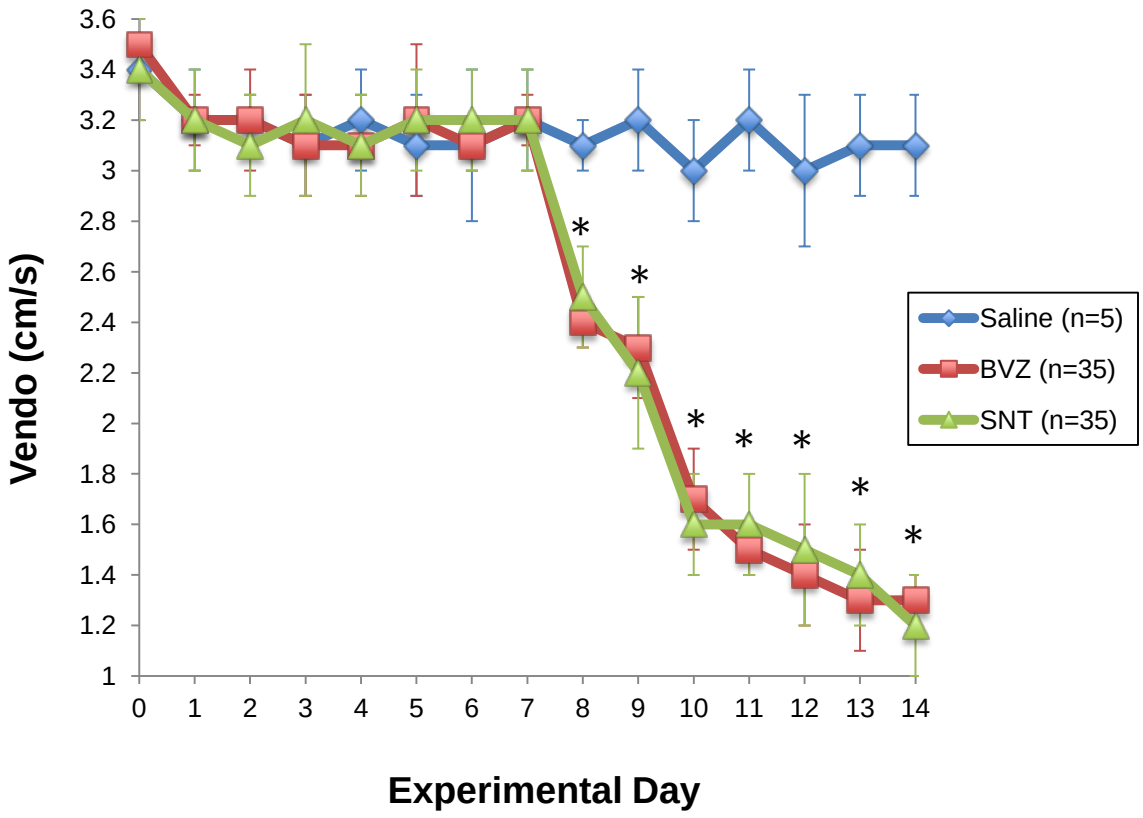


Figure 12: Peak endocardial systolic velocity [V_{endo} (cm/s)] in mice treated with saline, BVZ, and SNT. V_{endo} decreased in BVZ and SNT mice after 8 days. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; $V_{endo,}$ peak endocardial systolic velocity.

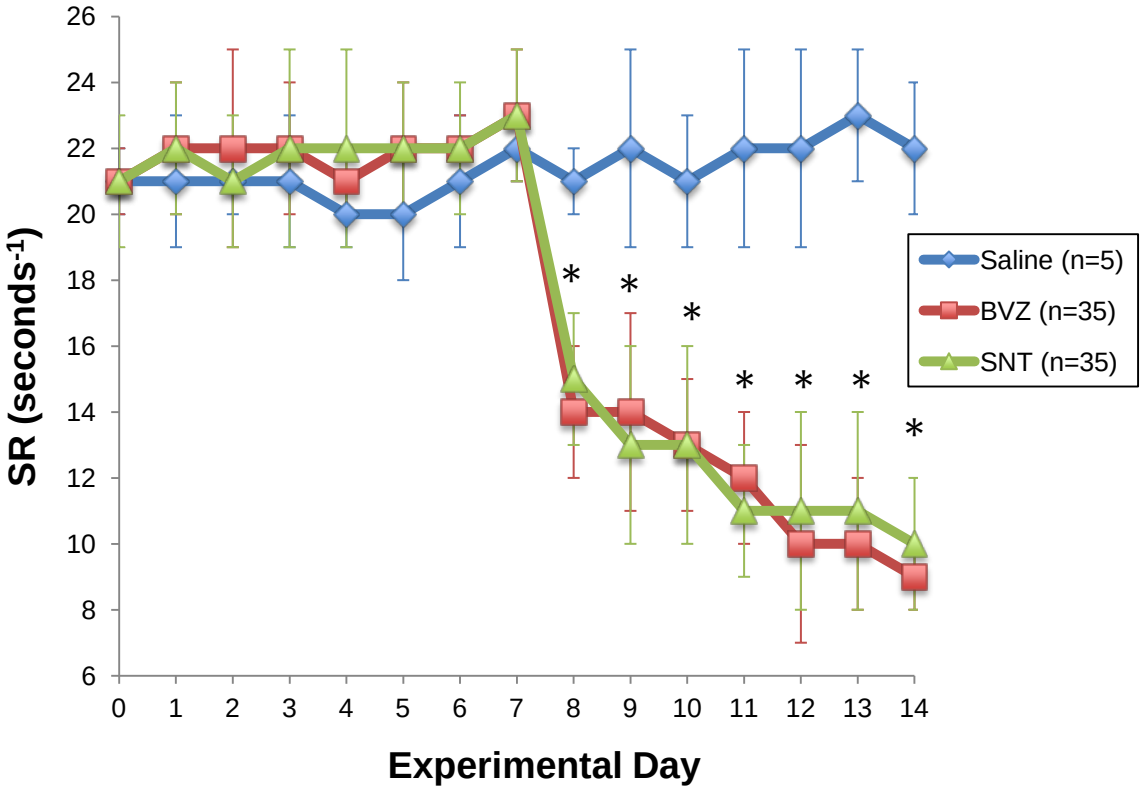


Figure 13: Strain Rate [SR (s^{-1})] values in mice treated with saline, BVZ, and SNT. SR decreases in BVZ and SNT mice after 8 days. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; SR, strain rate.

Cardiac Biomarkers: High sensitivity Troponin-I

All mice demonstrated non-detectable TnI values at baseline. At time of euthanasia, mice treated with DOX demonstrated an increase in TnI levels to 3.2±1.5 ng/ml. In mice treated with either BVZ or SNT, the TnI levels increased to 1.8±0.3 ng/ml and 2.3±0.4 ng/ml ($p<0.05$) respectively, at day 14 (Table 2). Blood samples collected on day 7 and day 10 demonstrated TnI levels of 0 ng/ml and did not show an increase in value.

Table 2: Cardiac Biomarker TnI

TnI (ng/mL)			
	Baseline	**Day 7**	**Day 14/Endpoint**
Saline (n=5)	0	0	0
DOX (n=20)	0	0	3.2±1.5*#
BVZ (n=35)	0	0	1.8±0.3*
SNT (n=35)	0	0	2.3±0.4*

*p=<0.05 compared to baseline
#Endpoint value: DOX mice did not survive the 14 day duration. Upon euthanization blood was immediately withdrawn and collected from the chest cavity.

Table 2: Cardiac biomarker TnI baseline values for all treatment groups were below detectable limits. Day 14 values are recorded as mean ± SEM in ng/mL. All DOX mice were euthanized before day 14, at their respective endpoints determined by V_{endo} <1.0 cm/s therefore, recorded values for DOX are from the endpoint when each animal was euthanized and blood was collected. DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib; TnI, Troponin I; $V_{endo,}$ peak endocardial systolic velocity.

Western Blot Analysis

Western blot analysis demonstrated evidence of apoptotic cell death in DOX, Bevacizumab, and Sunitinib treated animals (Figure 14). There were significantly increased levels of cleaved Caspase 3 amongst DOX, Bevacizumab, and Sunitinib treatment groups ($p<0.05$). As compared to saline treated animals, there was no significant evidence of an increase in either Bax or PARP expression in mice treated with Bevacizumab or Sunitinib. However animals treated with DOX (positive control) demonstrated a significant increase in Bax and PARP expression as compared to controls ($p<0.05$).

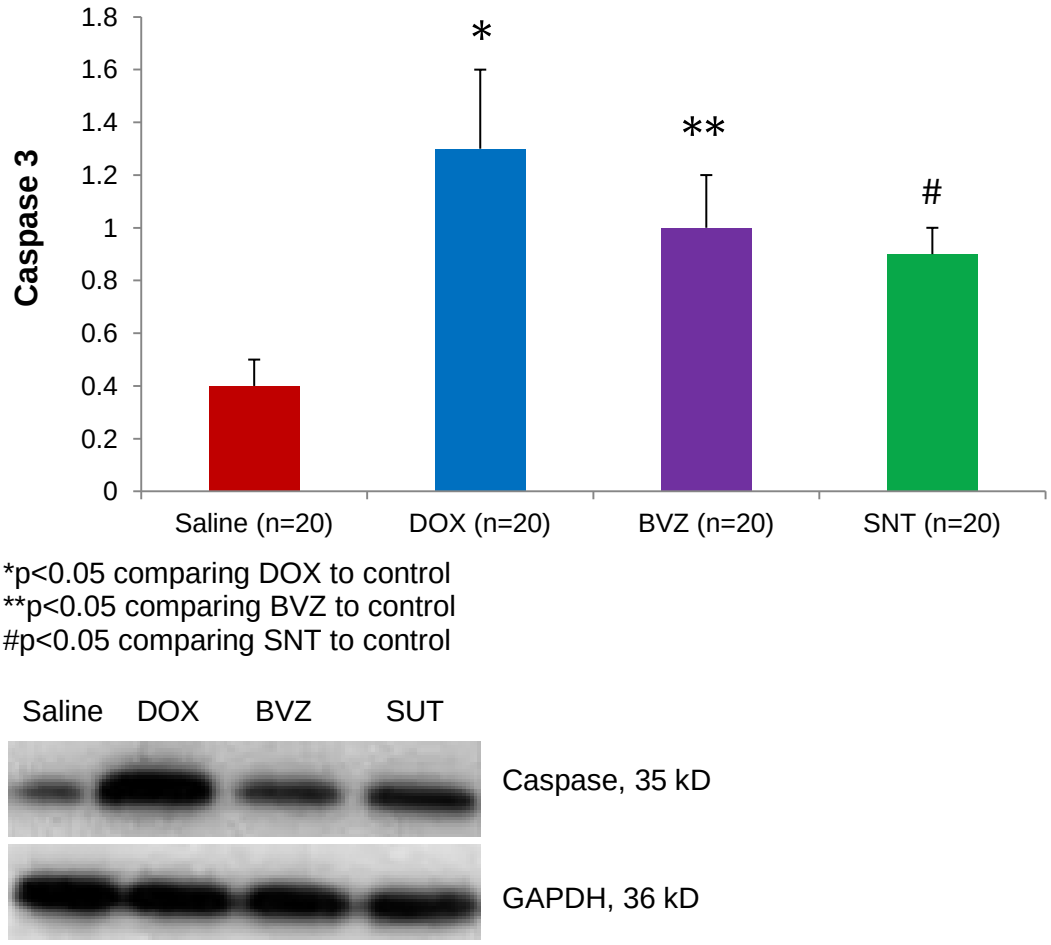


Figure 14: Graph demonstrating expression of proapoptotic protein Caspase 3 in mice treated with saline, DOX, BVZ, and SNT, in comparison to Caspase 3 expression in saline controls. Corresponding western blot demonstrating presence of Caspase 3 protein in treated mice. (Data for Bax and PARP proteins not shown). DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib; GAPDH, gluceraldehyde-3-phosphate dehydrogenase; PARP, Poly ADP ribose polymerase.

Electron Microscopy

Approximately 16, 000 cells were scanned from 3 randomly derived blocks of tissue and evaluated for sarcoplasmic reticulum dilation and loss of cell integrity. Significant differences were seen in loss of cell integrity frequency ($p<0.0001$) and severity ($p<0.0019$) between DOX versus BVZ and SNT treatment groups. There was no statistical differences between the BVZ and SNT treatment groups (Figure 15). However, as compared to control mice, BVZ and SNT treated animals demonstrated and increased loss of cellular integrity and myofibril disarray.

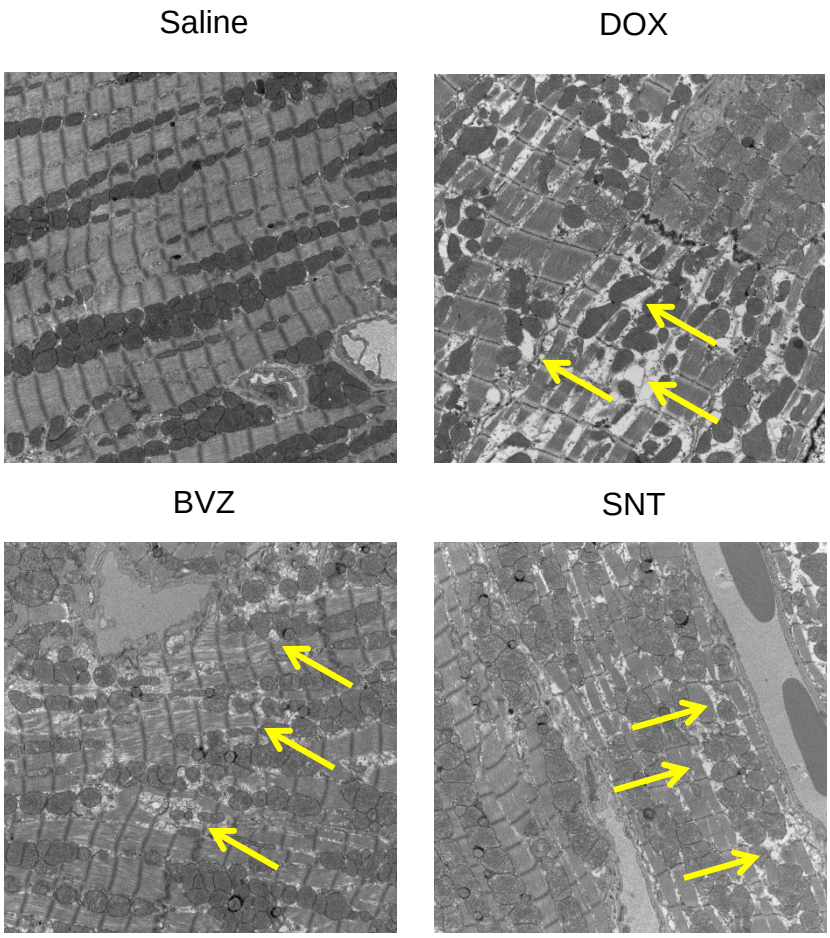


Figure 15: Electron microscopy of representative samples from mice treated with saline, DOX, BVZ, or SNT (5800X magnification). Arrows indicate disruption and loss of myofibrils of cardiomyocytes in the DOX, BVZ, and SNT treatment groups, as compared to controls. (Multiple t-Tests were performed for histological analyses). DOX, Doxorubicin; BVZ, Bevacizumab; SNT, Sunitinib.

Chapter 4: Discussion

Summary

The evolving discipline of Cardio-Oncology is concerned with the management of cancer patients with pre-existing cardiovascular disease and those patients who develop cancer drug induced cardiovascular complications. The enhanced understanding of the pathology of cancer has allowed for the recent development of targeted therapies including Bevacizumab and Sunitinib. Although effective against cancer in the clinical setting, these novel anti-cancer drugs are associated with an increased risk of developing cardiotoxicity. Therefore, it would be clinically useful to investigate novel early indices of LV systolic dysfunction in order to avoid the detrimental side effects of end-stage heart failure observed in this unique patient population. The aim of the current research proposal was to determine the potential use of cardiac biomarkers and echocardiographic parameters including TVI, for the early detection of drug-induced cardiotoxicity. In a murine model of Bevacizumab and Sunitinib mediated cardiotoxicity, we demonstrated: i) development of systemic hypertension; ii) cardiac biomarkers were unable to detect early LV systolic dysfunction; iii) novel use of echocardiographic parameters TVI and SR were able to detect early LV systolic dysfunction by day 8, as compared to traditional LVEF values which decreased at day 13; and iv) evidence of loss of cellular integrity and increased apoptosis in both Bevacizumab and Sunitinib treated animals.

Survival

The use of chemotherapeutic agents including DOX and TRZ has been shown to result in increased mortality in murine models. Together, Neilan *et al*.,[263] Jassal *et al*.,[255] and Walker *et al*.,[247] demonstrated a significant increased mortality rate in male mice

treated with DOX (20mg/kg) as early as day 5, with reduced overall survival by day 10. Our current study supports these findings in that mice treated with DOX (positive control) demonstrated a reduced survival rate as compared to mice treated with either Bevacizumab or Sunitinib. In congruence with a study by Chu *et al.*, which evaluated mice treated with Sunitinib and followed for a total of 12 days[197], the current study demonstrated a 100% survival rate among animals treated with novel targeted therapies, including BVZ and SNT. In comparison to conventional chemotherapies, novel targeted therapies including BVZ and SNT cause less cardiotoxicity, reflected by the decreased mortality rate observed in this animal model.

Bevacizumab and Sunitinib mediated Hypertension

The use of novel anti-angiogenic drugs including Bevacizumab and Sunitinib result in significantly increased blood pressure by approximately 25% as demonstrated in a variety of studies.[81, 85, 172] As previously described, increased signaling through the VEGF pathway has been identified as a key mediator of neoangiogenesis in malignant tumors.[267] Targeted agents such as Bevacizumab and Sunitinib block the VEGF signaling pathway, leading to decreased growth and metastasis of neoplasms. Although the pathogenesis of VEGF inhibitor-induced hypertension is incompletely understood, several hypotheses have been formulated. One theory involves the inhibition of VEGF, which leads to down-regulation of endothelial NO synthase expression, decreased synthesis of endothelial NO, resulting in vasculature constriction.[268] Reduced synthesis of NO consequently results in increased vascular tone and decreased sodium ion renal excretion, leading to elevated arterial pressure. In a rat model of VEGF treatment, Neagoe and colleagues[104] demonstrated a dose-related decrease in mean arterial BP through NO and prostacyclin

synthesis. Conversely, Chu *et al*. observed no change in blood pressure values, in animals treated with Sunitinib (40 mg/kg/d) and followed over a period of 12 days.[197] A complementary animal study by Giordano *et al*. successfully demonstrated the vital role of the VEGF pathway in maintaining cardiac function.[103] By inhibiting the pro-angiogenic pathway, Giordano and authors demonstrated a significant increase in systolic blood pressure in addition to thinning of the ventricular walls, and decreased contractile function in mice. A number of studies have also demonstrated vascular rarefaction as a vital mechanism in the development of hypertension. The inhibition of the VEGF pathway leads to a functional decrease in the number of arterioles and capillaries, resulting in increased peripheral vascular resistance and systolic blood pressures.[269] A study by Hutchins *et al*. evaluated a spontaneously hypertensive rat model, which exhibited rarefaction of arterioles and capillaries at 6 to 8 weeks of age.[270] In addition, Hansen-Smith *et al*. demonstrated that rats fed a high-sodium diet developed acute microvascular rarefaction after 3 days, suggestive of the advanced nature of rarefaction in the setting of hypertension.[271]

In our study, mice treated with Bevacizumab and Sunitinib developed significantly increased MAP as compared to baseline values. Animals treated with either BVZ or SNT demonstrated a significant increase in MAP ranging from 50-60% at day 14. This study is unique in that the development of hypertension was demonstrated in a mouse model of drug-mediated cardiomyopathy, adding to the existing data in hypertensive rat models. Experimental rat studies previously demonstrated a 30% increase in blood pressure from baseline values in the setting of oncologic therapies.[272] Furthermore, as various animal trials have demonstrated a chronic model of drug-induced hypertension, our acute *in vivo*

study verified the development of hypertension as early as 14 days following anti-cancer treatment. Although increased LV wall thickness is often associated with augmented blood pressure, our study demonstrated no change in LV wall dimensions. This may be due to the acute nature of our 14 day model, as hypertrophy is not typically observed until 4 to 6 weeks following the onset of hypertension. Therefore, future animal studies are warranted to elucidate the subsequent hemodynamic side effects of chronic administration of BVZ and SNT in a murine model.

Translating these basic science findings to the clinical arena, Hurwitz *et al*. demonstrated that of 402 total colorectal patients that received a combination of Bevacizumab and fluorouracil-based chemotherapy, 11% developed systolic BP ≥180 mmHg.[83] In comparison, of the 411 colorectal patients that were treated with chemotherapy alone, only 2% developed severe hypertension. On average, the median time to development of hypertension in humans is within one week following treatment initiation. A meta-analysis performed by Zhu *et al*., evaluated 1850 patients divided between seven trials, with various types of cancer, including colorectal cancer.[273] Treatment with Bevacizumab was associated with a significantly increased risk of all grades of hypertension, with incidence ranging between 3%-32% for the low-dose Bevacizumab (5 mg/kg), and between 18%-36% for the high-dose bevacizumab (10 mg/kg). Systolic BP ≥180 mmHg was also observed in 52 of 597 patients (9%) for low-dose and in 61 of 381 patients (16%) for high-dose Bevacizumab. A clinical trial by Mourad *et al*. measured dermal capillary densities in the dorsum of fingers using intravital video microscopy, for the assessment of microvascular endothelial function.[274] A total of 18 cancer patients receiving Bevacizumab underwent measurements at baseline

and 6 months post-treatment. The mean BP significantly increased following 6 months of therapy compared to baseline values, from 129 (±13)/ 75 (±7) mmHg to 145 (±17)/ 82 (±7) mmHg for systolic and diastolic BP, respectively ($p < 0.0001$). Mourad and authors also demonstrated a significant reduction in dermal capillary density and pilocarpine-induced vasodilation after 6 months of treatment, suggesting that treatment with Bevacizumab results in endothelial dysfunction and capillary rarefaction.

Similarly, clinical trials evaluating the efficacy of Sunitinib have also demonstrated the occurrence of drug-mediated hypertension. A study by Rixe *et al*., which evaluated patients receiving Sunitinib for the treatment of metastatic renal carcinoma, found that 23% of patients developed SBP ≥180 mmHg or severe hypertension.[275] Despite the SNT-mediated increases in BP levels observed in this oncologic patient population, various authors have demonstrated a correlation between the development of hypertension and increased response to cancer treatment. A study by Maitland *et al*. evaluated the efficacy of TKI's for the treatment of renal cell carcinoma.[276] Blood pressure values were recorded prior to the beginning of therapy and 6-10 days following drug administration. Both systolic and diastolic BP levels were significantly higher at day 6 or 10 with respect to basal measurements, indicative of the effective inhibition of the VEGF pathway. In support of these findings, Rixe *et al*. reported that in renal carcinoma patients receiving Sunitinib, a multivariate analysis grade ≥2 hypertension (SBP 160-179 mmHg) was an independent predictive factor of cancer response (OR= 2.33, p= 0.009), while SBP ≥180 mmHg correlated with greater overall survival (OR= 5.69, p=0.03).[275] However, other authors have found no relationship between this drug-mediated hypertension and response to SNT therapy. In a retrospective study by Wick *et al*., patients treated with

SNT demonstrated no significant difference in response rate and median survival based on the development of hypertension.[277] Similarily, Hurwitz *et al*. verified these findings by analyzing the predictive role of hypertension in 5,900 patients, enrolled between six separate trials, and all treated with a TKI.[278] With the exception of the AVF2107g trial,[83] there was no correlation found between BP changes and progression-free survival (PFS) in the remaining studies.[278] Therefore, the clinical value of the association between microvascular rarefaction, endothelial dysfunction, hypertension, and response to targeted drug treatment remains an area for future investigation.

Early Detection of Cardiotoxicity due to BVZ and SNT using Tissue Doppler Imaging

Traditionally, monitoring of carditoxicity involves the measurement of LVEF through the use of MUGA scans or echocardiography. The current clinical approach includes assessment of cardiac function at baseline, during, and immediately following completion of anti-cancer therapy.[279] Despite various proposed strategies, neither consensus nor evidence-based guidelines presently exist regarding the surveillance of cardiac function in cancer patients. In addition, there is a high degree of intra- and inter-observer variability in the analyses of conventional echocardiography, which limits its reproducibility and clinical usefulness.[249] Most importantly, LVEF measurement is a relatively insensitive parameter for the early detection of drug-mediated cardiotoxicity.[280] Once impaired systolic function is detected, with an LVEF <40%, irreversible cardiac injury may have already occurred, thereby precluding any chance of prevention.[281] The novel use of TVI has recently been established to supplement conventional echocardiography in the evaluation of myocardial function.[249] As compared to

conventional echocardiographic measurements, TVI-derived parameters are less influenced by loading conditions and provide more precise, reproducible analysis of both systolic and diastolic function. Furthermore, as traditional measurements of LVEF and FS may be influenced by blood pressure, TVI can detect early alterations in the LV myocardium and quantitatively assess the timing of LV contraction independently of hemodynamic variables.[249]

Several animal-based models have recently demonstrated the utility of TVI for the early detection of DOX mediated cardiac dysfunction. In a study performed by Jassal *et al*. in 2009, conventional echocardiographic parameters decreased at day 5 in mice treated with DOX alone.[255] Comparatively, measurements using TVI demonstrated a significant decrease in both V_{cndo} and SR as early as 24 hours following treatment, indicating that TVI was able to detect subtle changes in cardiac function prior to a reduction in conventional parameters. Extending these findings, a follow-up study by Walker *et al*. confirmed the role of TVI as a sensitive marker of DOX+TRZ induced cardiac dysfunction.[282] A reduction in LVEF was not observed until day 4 after treatment with the combination of DOX+TRZ; however V_{endo} and SR were able to detect early myocardial dysfunction within 24 hours, indicative of future cardiac impairment. Similarly, a study by Neilan *et al*. evaluated an animal model of DOX-induced cardiac injury and determined that TVI-derived parameters were able to detect LV dysfunction prior to alterations in conventional indices.[256] TVI measurements were predictive of the development of late cardiac dysfunction and mortality following administration of DOX.

In the setting of Bevacizumab and Sunitinib induced cardiotoxicity, the role of TVI for the detection of subtle changes in myocardial function has been unexplored. In the

current study, mice treated with Bevacizumab or Sunitinib demonstrated a decrease in conventional echo parameters, including LVEF and FS as well as an increase in LV cavity dimensions at day 13. In contrast, V_{endo} and SR decreased 5 days earlier, confirming that TVI is a sensitive, reproducible measure of early cardiac dysfunction in a murine model of Bevacizumab and Sunitinib mediated cardiotoxicity. A chronic model, which followed a subset of mice for a total of 28 days, demonstrated that early withdrawal of Bevacizumab and Sunitinib at day 8 results in only partial recovery of TVI parameters as compared to baseline. Additionally, in the 14 day study, V_{endo} and SR values decreased gradually as compared to LVEF, which demonstrated a dramatic reduction from day 12 to day 13. A plausible explanation for this is the regional injury pattern imparted by this drug-mediated cardiotoxicity. In the early stages of cardiac damage, normal functioning myocardial segments may compensate for injured segments, resulting in moderately preserved LVEF.[259] Furthermore, strain involves the averaging of multiple segment measurements, as compared to LVEF which includes a single tracing resulting in one measurement. Considering the lower variability of these novel imaging techniques, TVI may be a more feasible imaging modality for the early detection of subclinical LV systolic dysfunction.

A myriad of studies have demonstrated the clinical use of TVI for the early detection of cardiotoxicity of DOX+TRZ in the clinical setting. In a prospective study by Fallah-Rad *et al*. conducted between 2007-2009, 10 of 42 (24%) breast cancer patients developed DOX+TRZ mediated cardiomyopathy.[228] Both longitudinal and radial strain decreased as early as 3 months, whereas LVEF decreased at 6 months in women with TRZ mediated cardiotoxicity. A study by Sawaya *et al*. evaluated a total of 43 breast

cancer patients treated with a combination of anthracyclines and TRZ.[259] There was a significant decrease in LVEF by 8%, which was detected at 6 months post-treatment. In comparison, there was a reduction in both longitudinal strain (11%) and circumferential strain (15%), but not radial strain, at 3 months following treatment administration. Thus, the authors concluded that changes in longitudinal strain are able to predict the development of cardiotoxicity in patients treated with anthracyclines and TRZ. In the clinical setting of colorectal and renal cell cancer, little is known about the predictive role of TVI for the early detection of drug-induced cardiotoxicity. Chu *et al*. evaluated the prevalence of cardiac dysfunction in a population with metastatic GIST treated with Sunitinib.[197] The most common adverse cardiac event was New York Heart Association (NYHA) class III-IV congestive heart failure, which was reported among 8% of patients. Patients who received the recommended dosage of Sunitinib (50 mg/day, 4 weeks on and 2 weeks off cycle) also demonstrated a 1-5% reduction in LVEF per cycle, when followed over the first 24 weeks. In agreement with these findings, Motzer *et al*. reported a 10% rate of LV systolic dysfunction in renal-cell carcinoma patients treated for a median of 6 months.[189] Although the use of velocity-derived indices in this unique population remains novel, the current study demonstrates the potential of TVI parameters in detecting subtle changes in cardiac function, prior to conventional measurements currently implemented for the detection of drug-induced cardiotoxicity in the clinical setting.

Cardiac Biomarkers: Troponin I

In recent years, there has been increasing interest in the use of cardiac biomarkers to detect early cardiac damage in the cancer setting. Challenges exist in the assessment of

drug-induced cardiotoxicity due to the considerable reserve of cardiac function observed in the average healthy heart. Thus, significant damage may occur prior to the onset of symptoms, resulting in irreversible cardiac dysfunction. Basic science and clinical studies have frequently assessed the role of cardiac biomarker changes including, troponin, C-reactive protein (CRP), and brain natriuretic peptide (BNP), in the oncologic therapy setting. Cardiac troponins including TnI and TnT, are cardiac regulatory proteins, which are involved in the calcium-mediated interaction of actin and myosin. In comparison, cardiac troponin I is normally undetected outside the myocardium, whereas cardiac troponin T is mildly expressed in skeletal muscle. Troponins are superior in the measurement of myocardial dysfunction due to the near absolute cardiac tissue specificity, as well as increased sensitivity in detecting subtle necrosis, which is disallowed by less sensitive biomarkers such as creatine kinase.[283] There are several pathophysiological mechanisms which can lead to troponin elevations, including a mismatch between myocardial oxygen demand and supply in the absence of flow-limiting epicardial stenosis, prolonged myocardial ischemia, direct myocardial damage, myocardial strain, and chronic renal insufficiency.[284] In addition to the diagnosis of acute coronary syndromes, the use of troponins have extended to a wide range of diverse cardiac pathologies, including left ventricular hypertrophy, heart failure, pulmonary embolism, blunt trauma, sepsis, stroke, renal insufficiency, and cardiotoxicity associated with anticancer drugs.[231, 285, 286]

The role of cardiac troponins as markers of early anthracycline-induced cardiotoxicity was initially reported by Seino *et al*., in a model of spontaneously, hypertensive rats.[287] In corroboration, a study by Bertinchant *et al*. evaluated cardiac TnI

and TnT as early markers of myocardial damage, in a rat model of Doxorubicin-induced cardiomyopathy.[288] A significant increase in cardiac TnT was observed in animals treated with Doxorubicin after cumulative doses of 7.5 and 12 mg/kg, as compared to baseline ($p<0.05$). Specifically, cardiac TnT values were significantly greater in animals administered 12 mg/kg of Doxorubicin as compared to the 7.5 mg/kg dosage. The authors concluded that cardiac TnT was most accurately able to detect myocardial damage, as assessed with echocardiography and histological changes. In addition, several studies performed by Herman *et al*. have confirmed the utility of serial serum cardiac TnT measurements for the early detection of DOX-induced cardiomyopathy.[289] Spontaneously hypertensive rats treated with between 7 to 12 mg/kg of Doxorubicin and followed for 12 weeks, demonstrated increased serum levels of cardiac TnT (0.03 to 0.05 ng/mL) and myocardial lesions. The average cardiac TnT levels were also associated with the cumulative dose of Doxorubicin (12 mg/kg Doxorubicin). Collectively, these studies illustrate the strong correlation between increased serum levels of cardiac TnT and severity of dose-dependent, anthracycline induced myocardial injury.[289, 290]

The use of cardiac troponin for the prediction of ensuing drug-mediated cardiotoxicity has been minimally investigated in the setting of Bevacizumab and Sunitinib. Chen *et al*. examined the cardiotoxic effects of Bevacizumab and 5-FU in a mouse model of cardiomyopathy. Animals administered either Bevacizumab (10 mg/kg) or Bevacizumab (10 mg/kg) plus 5-FU (15 mg/kg) demonstrated substantially higher TnI serum concentrations, as compared to mice treated with EndoCD [endostatin cytosine deaminase (60 mg/kg)] plus 5-fluorocytosine [5-FC (500 mg/kg)] or 5-FU (15 mg/kg) alone,[87] verifying the role of TnI as an early cardiotoxic marker in a chronic (6 month

follow-up) animal model. Our specific study is unique in the evaluation of Bevacizumab mediated cardiotoxicity in an acute murine model. To our knowledge, a basic science model investigating the predictive value of troponin, in the setting of Sunitinib-mediated cardiotoxicity has yet to be established. The objective of our current study was to evaluate the predictive role of cardiac TnI in the setting of Bevacizumab and Sunitinib mediated cardiotoxicity. Our basic science study results indicated elevated TnI levels at day 14 in both the Bevacizumab and Sunitinib treated groups. The ability of TnI to serve as an early predictive marker was not validated, as measurements yielded no change in hsTnI at day 7. Therefore, the current findings are novel in that augmented troponin levels were demonstrated in a model of targeted therapy at day 14; however our study was unable to confirm the predictive role of TnI for the early detection of Bevacizumab and Sunitinib mediated cardiotoxicity.

Numerous clinical trials have evaluated the use of cardiac biomarkers for the identification of cancer patients at increased risk of developing cardiotoxicity. A recent clinical study performed by our group evaluated the utility of cardiac biomarkers for the early prediction of LV systolic dysfunction in HER-2 positive breast cancer patients treated with the combination of DOX+TRZ.[228] Of the total 42 breast cancer patients enrolled, 10 (25%) women developed DOX+TRZ induced cardiotoxicity. Cardiac biomarkers including TnT, CRP, and BNP were not able to predict early LV systolic dysfunction in these patients. In contrast, through the frequent sampling of TnI, Cardinale *et al*. identified a subset of women at high risk of DOX+TRZ mediated cardiac dysfunction, prior to a decrease in LVEF.[229] Confirming these findings, a study by Ky *et al*. recently evaluated the association between various biomarkers and the development of

cardiotoxicity in HER-2 positive breast cancer patients treated with DOX+TRZ.[246] Levels of TnI, CRP, and myeloperoxidase (MPO) increased from baseline to 3 months ($p<0.05$), while considerable changes in TnI were associated with a greater risk of cardiotoxicity. Large, interval changes in TnI and MPO were associated with 47% increased risk of developing cardiotoxicity following chemotherapy administration. Finally, Chu *et al*. demonstrated that among GIST patients receiving therapeutic Sunitinib, 18% of the cohort exhibited abnormal troponin levels.[197] Further clinical studies evaluating the potential role of cardiac biomarkers for the early detection of Bevacizumab and Sunitinib mediated cardiotoxicity in the clinical setting are warranted.

Mechanisms for BVZ and SNT mediated Cardiotoxicity

Various basic science and clinical studies have demonstrated the cardiotoxic effects caused by anti-angiogenic therapies; however the precise underlying mechanisms have yet to be fully elucidated. Previously, the actions of anthracycline-mediated apoptosis have been suggested to result in cardiac remodeling, as seen in the chemotherapy setting.[200,263] Anthracycline-derived cardiotoxicity, a typically irreversible event, is associated with total cumulative drug dose and changes in myocardial ultrastructure, including vacuolization and cardiomyocyte loss.[291] In contrast, cardiotoxicity induced by targeted drug therapies including Trastuzumab, have demonstrated partial or complete reversibility with no significant ultrastructural changes.[250] The current study of Bevacizumab and Sunitinib mediated cardiotoxicity demonstrates a comparable decrease in echocardiographic parameters, however reduced TVI was detected as early as day 8 compared to day 13. Several animal models have demonstrated microscopic alterations in the myocardium as a direct result of anthracycline therapy. Evaluation of tissue using

light microscopy demonstrated that mice treated with DOX displayed extensive myofibrillar degradation and cellular vacuolization, in two separate studies performed by Jassal and Walker.[247, 255] Importantly, mice that received the combination therapy of DOX+TRZ demonstrated significantly more myocardial damage as compared to those mice treated with DOX alone. In the setting of targeted therapy, Chu and colleagues demonstrated mitochondrial swelling and degenerative changes in cardiomyocytes using transmission electronmicroscopy, including membrane whorls and effaced cristae, in animals treated with 40 mg/kg/d of Sunitinib.[197] The current study demonstrates there was a significant loss of cell integrity in DOX treated mice (positive control), as well as significant frequency and severity in loss of cell integrity observed in Bevacizumab and Sunitinib treated animals. These findings are indicative of cellular damage caused by impaired ATP generation due to mitochondrial dysfunction. In the current study however, there was no fibrosis observed with electron microscopy, which suggests the potential reversibility of the cardiomyocyte damage imparted through Bevacizumab and Sunitinib therapy.

Previous animal trials have indicated that the combined administration of DOX+TRZ therapy results in a synergist apoptotic effect, leading to increased cell death. A study by Jassal *et al*. evaluated the extent of cardiac apoptosis associated with the combination of DOX+TRZ therapy.[255] In a wild-type, animal model, co-administration of DOX+TRZ induced the greatest degree of apoptosis at day 10, compared to either agent alone. At the basis of these findings, an increase in PARP cleavage, activation of Caspase 3, and alteration in Bax/Bcl-X_L ratio was demonstrated in the hearts of chemotherapy treated animals. Walker *et al*. confirmed these apoptotic findings in a therapeutic model of DOX

(20mg/kg), TRZ (10mg/kg), and combined administration of both drugs.[282] The degree of cardiac dysfunction was found to be greatest among animals co-treated with DOX+TRZ than with either agent alone, and directly correlated to an increased apoptotic response measured by the increased Bax-to-Bcl-X_L ratio seen at day 10.

The off-target actions of novel drug agents such as Bevacizumab and Sunitinib are proposed to result in cardiac impairment. Anti-VEGF agents target several regulatory proteins including the ribosomal S6 kinase (RSK) family and AMPK, which have been reported to transduce pro-survival signals in the heart.[292, 293] Apoptosis is initiated through mitochondrial dysfunction, which is regulated by the Bcl-2 protein family. Both pro-apoptotic and anti-apoptotic proteins, including Bax, and Bcl-X_L respectively, comprise the Bcl-2 family. In the presence of anti-cancer drugs, pro-apoptotic proteins including Bax are activated, resulting in the formation of Bax homodimers that translocate from the cytoplasm to the mitochondria.[294] The homodimerized Bax then acts on voltage-gated anion channels located in the outer membrane of mitochondria, which leads to the release of cytochrome *c*.[295] Once released into the cytosol, cytochrome *c* forms an essential part of the apoptosis complex "apoptosome," which is composed of Apaf-1, procaspase-9, as well as cytochrome *c*. Formation of the apoptosome results in the activation of caspase-9, which in turn leads to cleavage of downstream effector caspases, including caspase-3 and caspase-7.[296, 297] The activation of caspase-3 and caspase-7 results in the cleavage of PARP and DNA fragmentation (Figure 3).

Animal models, including the study performed by Chu *et al*., demonstrated that Sunitinib targets the mitochondria in cultured cardiomyocytes, resulting in cytochrome-C release.[197] Corroborating these findings, the current study demonstrated a significant

increase in caspase-3 in Bevacizumab and Sunitinib treated animals, as well as a nonsignificant increase in the apoptotic proteins Bax, and PARP, as compared to controls. Although the increase in Bax and PARP was not significant in the treatment groups, increases in caspase-3 were significant in both Bevacizumab and Sunitinib treated mice. From these current results, it is logical to propose that the apoptotic events induced by Bevacizumab and Sunitinib are not solely mediated through the Bax/Bcl pathway. In support of this hypothesis, Hasinoff *et al*. demonstrated that in ventricular myocytes treated with Sunitinib, Bax levels were not significantly changed, indicative of the inactivity of this pathway in the induction of TKI-induced apoptosis.[298] However, this group also demonstrated that levels of caspase-3 and caspase-7 rapidly increased in myocytes following Sunitinib treatment, allowing the authors to conclude that the caspase pathway may play a significant role in mediating cardiotoxicity. Although a downstream target of caspase, decreased levels of PARP demonstrated in the current study may be attributed to limited DNA damage. PARP is involved in the regulation of programmed cell death mediated through the production of DNA strand breaks.[299] The activation of PARP results in poly-ADP-ribosylation of key DNA repair proteins, through the use of NAD^+ as an energy source. In the setting of minimal DNA damage, as may be the case with novel targeted TKI's, this physiological mechanism is capable of repairing the injury, resulting in the survival of the damaged cells.[300] Therefore, due to the decreased DNA damage caused by novel targeted therapies, levels of the apoptotic PARP protein may be reduced following the administration of either Bevacizumab or Sunitinib. In the future, additional basic science and clinical trials are warranted to thoroughly elucidate the underlying mechanisms involved in the cellular dysregulation and cardiomyocyte

apoptosis observed in this unique population of drug-mediated heart failure.

Limitations

There are several limitations of this current study that must be addressed. First, we only characterized drug-induced cardiac dysfunction in an acute male murine model of Bevacizumab and Sunitinib mediated heart failure over a total of 14 days. As these drugs are administered to patients over a period of several months, it would be useful to design a chronic murine model of Bevacizumab and Sunitinib mediated cardiac dysfunction, which more closely mimics the clinical setting. In the future, we will accomplish this through the serial administration of Bevacizumab and Sunitinib over a period of 6 weeks, with serial measurements of echocardiography, biomarkers, and BP, on a weekly basis for all animals. Second, we primarily evaluated changes in cardiac TnI. Among patients treated with targeted therapies, numerous cardiac biomarkers have been identified as predictors of ensuing cardiac dysfunction. Thus, the evaluation of cardiac biomarkers, including cardiac TnT and BNP, should be investigated for their predictive role in the heart failure setting. Finally, hemodynamic assessment and histological analysis was only performed at day 14 of the study. In the clinical setting, presentation of augmented BP levels occurs shortly after the initiation of targeted drug therapy. Therefore, it would be valuable to serially evaluate BP indices as well as ultrastructural changes on electron microscopy at day 8, to corroborate the TVI findings demonstrated in this model of Bevacizumab and Sunitinib induced cardiomyopathy.

Future Directions

Although we are confident that echocardiography will be able to detect early diastolic dysfunction in mice receiving either BVZ or SNT treatments, a possible area for future studies is the use of invasive hemodynamics to measure diastolic parameters. In the clinical setting reduced diastolic function is a precursor for ensuing HF, thus early detection of diastolic impairment may result in decreased morbidity and mortality rates among BVZ- and SNT-treated patients. Additionally, in a murine model of Bevacizumab induced cardiomyopathy, decreased NO production and reduced angiogenesis result in cardiomyocyte loss and heart failure. As our group has extensive experience with NOS3 in various animal models, it is plausible to study the effects of BVZ on mice with a congenital absence of NOS3 to determine whether these cardiomyocytes are protected from the cardiotoxic effects of this anti-cancer drug. Finally, our group is currently translating our basic science findings to our ongoing clinical trial, which involves the recruitment of colorectal and renal cell cancer patients, in collaboration with the Mayo clinic. We hypothesize that through the use of cardiac biomarkers and TVI/strain imaging, we will be able to detect subclinical alterations in cardiac function prior to a decrease in traditional LVEF in colorectal and RCC patients treated with BVZ or SNT, respectively. Essentially, the purpose of this clinical trial is to translate our current findings from this animal model to the human arena, for the potential prevention and treatment of Bevacizumab and Sunitinib induced cardiotoxicity, before the progression to end-stage heart failure.

Clinical Implications

The surveillance of cardiac function among cancer patients is currently obtained through the use of MUGA scans and echocardiography. The conventional measurement of LVEF remains the single most important diagnostic tool used in the clinical setting. However, once a reduction in LVEF is detected, significant cardiac injury may have already occurred. Therefore, it would be clinically useful to non-invasively detect subtle early changes in cardiac function, prior to significant cardiac injury through the implementation of cardiac biomarkers and TVI, in patients treated with Bevacizumab or Sunitinib. Our study is novel in that it suggests the potential role of TVI as an early predictor of cardiac injury, which may allow for the prevention and treatment of this drug-induced cardiotoxicity. Thus, in a cancer population of developed heart failure, one may consider adjusting the dosage of anti-cancer drugs Bevacizumab or Sunitinib, and/or administrating cardioprotective agents, essentially preventing the development of irreversible cardiac dysfunction.

Conclusion

For the first time, our novel study demonstrates that TVI is able to serve as an early predictor of cardiac dysfunction in an acute murine model of Bevacizumab and Sunitinib induced cardiomyopathy. In each individual animal that demonstrated a decrease in V_{endo}, a concomitant reduction in LVEF was observed by day 13. It is important to note that no single mouse that demonstrated a drop in V_{endo} or SR had a preserved EF throughout the 14 day study. Future research is required to thoroughly understand the subcellular mechanisms involved and to investigate the potential of cardioprotective agents in mitigating the cardiotoxic effects caused by these anti-cancer drugs, in a more clinically relevant murine model of Bevacizumab and Sunitinib mediated cardiac dysfunction.

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