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Project Title: Avastin and Sutent Induced Cardiotoxicity Study (ASICS): Early Detection of Bevacizumab and Sunitinib Mediated Cardiotoxicity

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SUMMARY: (no more than 250 words single spaced)

BACKGROUND: Bevacizumab (BVZ) and Sunitinib (SNT) are two novel treatments which are used in the setting of colorectal cancer and renal cell carcinoma, respectively. While both agents are known to suppress tumour cell progression, they have been associated with the onset of drug-induced cardiotoxicity. Early, non-invasive methods of detecting cardiac dysfunction would be useful for preventing end stage heart failure.
OBJECTIVE: To determine if cardiac biomarkers and/or tissue Doppler echocardiographic techniques may allow for early detection of BVZ and SNT induced LV systolic dysfunction in an acute murine model.
METHODS: A total of 75 wild type (WT) C57Bl/6 male mice were treated with either 0.9% saline, BVZ, or SNT and followed for a total of 14 days. Serial echocardiography, hemodynamic monitoring, and cardiac biomarkers were performed over the study period after which all mice were euthanized for histological and biochemical analyses.
RESULTS: In WT mice receiving BVZ or SNT, left ventricular ejection fraction (LVEF) values decreased from 75±3% at baseline to 52±2% and 49±1% at day 13, respectively. In contrast, there was a significant decrease in tissue Doppler imaging (TDI) parameters by day 8, indicative of early LV systolic dysfunction. Hemodynamic monitoring at day 14 indicated the development of hypertension following BVZ or SNT treatment. Histological and biochemical analyses demonstrated loss of cell integrity and elevated caspase-3, a marker of cell apoptosis, in mice receiving BVZ or SNT.
CONCLUSION: In an acute murine model, TDI echocardiographic indices were earlier indicators of BVZ and SNT mediated cardiotoxicity.

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INTRODUCTION

In Canada, cardiovascular disease and cancer represent two equally significant health concerns for the public. Currently, the treatment for various cancers incorporates a multi-faceted approach which includes surgery, external beam radiation, and chemotherapy. However, recent medical literature has identified an increasing occurrence of cardiac disease in cancer patients due to chemotherapy treatment.[1] The introduction of novel targeted biological drug therapies including the monoclonal antibody Bevacizumab (BVZ) and the tyrosine kinase inhibitor Sunitinib (SNT) have improved the long term survival in patients with colorectal cancer (CRC)[2] and renal cell cancer (RCC)[3], respectively. Despite the beneficial effects of BVZ and SNT in reducing tumour burden, however, there is an increased risk of cardiotoxicity. Importantly, the occurrence of cardiac dysfunction, plays a deleterious role in terms of the prognosis and subsequent therapeutic options of patients affected with cancer.[4] Although clinical trials suggest that the prevalence of BVZ and SNT mediated cardiotoxicity is up to 10%, [5,6] recent real world studies demonstrate a risk closer to 25%.[7,8]

Presently, CRC is the third leading cause of cancer-related illness in Canada with an estimated incident rate of 24, 400 new cases per year and accounts for up to 9,300 deaths per year.[9] For treatment of solid tumours including CRC, Bevacizumab (Avastin) has emerged 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), leucovorin, oxaliplatin and irinotecan.[10] BVZ is a IgG_1 monoclonal antibody inhibiting tumour angiogenesis that targets the vascular endothelial growth factor isoform (VEGF-A).[11] However, BVZ is associated with unanticipated cardiotoxic side effects likely due to alterations in endothelial nitric oxide (NO) production and/or impaired myocardial angiogenesis (Figure 1). Previous VEGF knockout studies have supported the theory, in which cardiomyocyte-specific VEGF knockout mice demonstrated diminished contractile function likely due to cardiac hypovascularity.[12] In the clinical setting, hypertension and congestive heart failure (CHF) develop as concerning cardiac side effects associated with BVZ administration.[13] In a retrospective study evaluating the prevalence of BVZ-mediated cardiotoxicity in CRC patients at Cancer Care Manitoba (CCMB) from 2010-2011, roughly 1 in 4 patients developed LV systolic dysfunction.[14]

In 2014, RCC remains a significant health issue with approximately 6,000 new cases per year and 1,750 related deaths reported in Canada.[9] In the management of advanced RCC disease, several targeted agents, including Sunitinib (Sutent), have recently been incorporated into the therapeutic program. It is well known that malignant cell transformations result in spontaneous loss of tyrosine kinase regulation contributing to tumor neoangiogenesis. Sunitinib (Sutent) is a multi-targeted receptor tyrosine kinase inhibitor (TKI) with established efficacy in the setting of metastatic renal cell carcinoma (RCC). Specifically, SNT prevents neoplastic vasculature growth and maintenance by inhibiting vascular endothelial growth factor receptors (VEGFR) 1-3. Unfortunately, SNT may cause cardiomyocyte apoptosis and LV systolic dysfunction initiated by VEGFR inhibition. Animal models suggest SNT-mediated cardiotoxicity is related to impaired myocardial angiogenesis, increased oxidative stress, and/or inhibition of adenosine monophosphate-activated kinase (AMPK) (Figure 1). [15] In a retrospective study of patients receiving TKI therapy, approximately 34% developed an adverse cardiac event, defined as the occurrence of increased cardiac enzymes, symptomatic arrhythmia, new left ventricular dysfunction, or acute coronary syndrome.[16]

In spite of the survival benefits conferred by BVZ and SNT in patients with CRC or RCC, respectively, the significant risk of cardiotoxicity necessitates further investigation. In the clinical management of cancer patients treated with anti-cancer drugs including BVZ and SNT, serial

assessment of left ventricular ejection fraction (LVEF) using multi-gated acquisition scan (MUGA) and transthoracic echocardiography (TTE) remains the exclusive method in monitoring the onset of cardiac dysfunction in this patient population.[17, 18] However, compensatory myocardial reserve enables adequate ventricular output even in the presence of dysfunctional cardiomyocytes and thus, extent of cardiac injury is often undervalued by traditional LVEF assessment despite its prevalent use in the clinical setting.[19]

While normal cardiac systolic function is defined by an LVEF > 60%, heart failure can be identified with an LVEF < 40% which may potentially lead to irreversible cardiac injury. The Cardiac Review and Evaluation Committee has previously established diagnostic criteria outlining the drug-mediated cardiac dysfunction as one or more of the following: 1) cardiomyopathy characterized by a global reduction in LVEF; 2) symptoms consistent with CHF; 3) clinical signs consistent with CHF; and/or 4) a decline in LVEF ranging between 5-55% as compared to baseline accompanied by clinical findings of CHF or a decline in LVEF ranging between 10-55% in the absence of CHF-related signs and/or symptoms.[20] Unfortunately, the use of traditional serial LVEF assessments in the early detection of cardiac dysfunction is inadequate as a decline in LVEF values represent a late marker of considerable myocardial injury. Specifically, LVEF reduction is associated with persistent LV systolic functional impairment as high as 58% in patients receiving cardiotoxic agents, despite intervention.[21] In contrast, cardiac biomarkers and novel echo parameters have emerged as more sensitive and specific tools in the early recognition of anti-cancer drug mediated cardiotoxicity.[22,23]

The utility of cardiac biomarkers have been well documented as sensitive markers of early LV systolic dysfunction in individuals with heart failure, correlating to increased morbidity and mortality.[24,25] Specifically, cardiac troponins, C-reactive protein (CRP), and brain natriuretic peptide (BNP) have gained acceptance as valid diagnostic predictors for a broad range of cardiac pathologies. Troponin I (TnI) has been used as a biochemical index of myocardial damage and has recently been identified as a sensitive indicator of chemotherapy induced cardiotoxicity in both the basic science and clinical setting.[26] Principally, mammalian studies performed in both mice and rats have demonstrated that cardiac troponins are a reliable biomarker of cardiotoxicity associated with anticancer drugs.[27] In the breast cancer setting, previous clinical studies have demonstrated the value of TnI as a risk marker for the development of significant and prolonged left ventricular dysfunction following high-dose anthracycline therapy.[4] However, current literature has yet to establish if cardiac biomarker monitoring is a feasible diagnostic tool in predicting early evidence BVZ and SNT cardiac toxicity.

Likewise, novel echocardiographic techniques of tissue Doppler Imaging (TDI) may accurately detect early myocardial injury prior to changes in traditional LVEF assessment.[28,29] In echocardiography, TDI refers to a modified blood-flow Doppler which enables identification of tissue-derived, high amplitude and low velocity Doppler signals.[30] As such, this imaging modality allows for the evaluation of ventricular wall and mitral annulus velocities during the entire cardiac cycle.[31] TDI indices include myocardial tissue velocity imaging (TVI) and strain rate (SR).[32,33] In murine models of anthracycline-related cardiotoxicity, TDI indices (V_{ENDO}, SR) predicted early LV systolic dysfunction by 4 days as compared to traditional LVEF assessment.[34] In addition, TDI and SR have also been previously validated in the early detection of chemotherapy-induced cardiomyopathy in breast cancer patients.[21,29,30,34] Meta-analysis of myocardial strain rate imaging established that TDI-based strain, longitudinal SR of the basal interventricular septum consistently demonstrates a reduction between pre-therapy and low doses of anthracyclines.[21] It remains to be seen if novel non-invasive

echocardiographic techniques can detect early cardiac dysfunction in the setting of BVZ or SNT induced cardiotoxicity.

OBJECTIVE

The primary objective of the study is to determine if cardiac biomarkers and/or tissue Doppler echocardiographic techniques may allow for ***early detection*** of LV systolic dysfunction in an acute murine model of BVZ and SNT mediated cardiotoxicity prior to conventional assessment of LVEF.

METHODS

A. ANIMAL MODEL

All animal handling procedures were performed in accordance with the guidelines published by the Canadian Council on Animal Care. All experimental procedures, including drug administration and longitudinal echocardiographic studies, were approved by the University of Manitoba Animal Care Committee.

An acute murine model of chemotherapy induced cardiomyopathy was studied using a total of 75 wild-type C57Bl/6 male mice, randomly assigned to one of the following three groups: i) Control (0.9% saline i.p., n=5); ii) Bevacizumab (BVZ 10 mg/kg i.v., n=35); or iii) Sunitinib (SNT 40 mg/kg/d orally, n=35) and followed for 14 days (Figure 2). BVZ was administered as a single parenteral dose at the start of the study after baseline echocardiographic study. SNT was administered daily via oral gavage for a total of fourteen doses spanning the length of the study. Treatment dosages of both BVZ and SNT were comparable to those observed in the clinical setting.[5,6] Following the initiation of the experimental protocol, serial echocardiography was performed daily for a total period of fourteen days. At the end of the study, mice were euthanized for histological analysis.

B. MURINE ECHOCARDIOGRAPHY

In vivo assessment of cardiac structure and function was evaluated on awake mice at baseline and daily for a total of 14 days via TTE with TVI and SR assessment using both a 13-MHz probe (Vivid 7, GE Medical Systems, Milwaukee, WI, US) and a 40-MHz probe (Vevo 2100, Visualsonics).[28] Each echocardiographic study was performed in both the parasternal long axis view and parasternal short axis view. The assessment of echocardiographic parameters was performed offline using EchoPAC PC software (version 11.2, Vivid 7, GE Healthcare, Milwaukee, WI, US).

A 2D parasternal long axis echocardiographic view (PLAX) of the left ventricle was used for estimations of the LVEF based on interpreted tracings of the end diastolic (LVEDV) and end systolic (LVESV) volumes. M-Mode echocardiography in the 2D parasternal short axis view (SAX) was used for the measurement of several LV cavity dimensions including: interventricular septal diameter (LVSd), left ventricular end-diastolic diameter (LVIDd), left ventricular posterior wall diameter (LVPWd), and left ventricular end-systolic diameter (LVIDs). LV fractional shortening (FS) was also calculated using the M-Mode LVSd and LVIDd parameters. All calculations (LVEF, FS) were automated by the GE EchoPAC PC software following the evaluation of the basic dimensions acquired from manual offline image analysis.

TDI assessment was carried out at the level of the papillary muscles using a 2D parasternal short axis view at a frame rate of 483 frames per second. Peak endocardial systolic velocity (V_{ENDO}) was measured in a 0.2 x 0.2 mm region in the posterior wall of the endocardium. Radial strain rate (SR) was measured over an axial distance of 1 mm (width 0.6

mm). All values obtained during tissue Doppler imaging averaged across five consecutive cardiac cycles.

C. HEMODYNAMIC MONITORING

Mean arterial blood pressure (MAP) and heart rate (HR) was measured non-invasively at baseline and day 14 using a murine tail cuff apparatus, as previously described (CODA System, Kent Scientific, Torrington, CT). [35] The monitoring procedure was performed on awake mice acclimatized by a 30°C heated holding platform. At each time point, hemodynamic data was collected a total of five times for each subject with one minute resting intervals taken between readings.

D. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

In vivo murine blood samples were collected at baseline, day 7 and day 10 via the jugular vein venipuncture. Following euthanasia of surviving mice at the end of the fourteen day study period, blood samples were pipetted directly from the mice thoracic cavity for biochemical analysis. Immediately following collection at each time point, blood samples were placed on ice and centrifuged at 1300g for a fifteen minute period to isolate the serum sample which was subsequently stored at -80°C for later analysis.

Mouse-specific ELISA analysis (Life Diagnostics, Inc., West Chester, PA, US) of the serum samples were performed to identify concentrations of High Sensitivity Troponin I (hsTnI). Following a 4x dilution process, absorbance values of the experimental samples were recorded at 450nm and compared against a standard curve prepared via serial dilution of hsTnI standard solution.

E. HISTOLOGICAL ANALYSIS

At the end of the study, all mice were euthanized (55 mg/kg pentobarbital; i.p.) and their hearts were collected and halved. The atria were subsequently removed and only left ventricle (LV) tissue samples were used for further analysis. One half of the LV was immediately flash frozen using liquid nitrogen and stored for subsequent biochemical analysis. The remaining half of the LV was assessed under electron microscopy for the degree of cardiomyocyte damage.[36]

LV sections destined for histological examination were evaluated by electron microscopy (EM). After fixation of cardiac tissue, samples were dehydrated in ascending concentrations of ethanol and embedded in Epon 812 using standard techniques.[37] A Philips CM12 transmission electron microscope (TEM) offering 5800x magnification was used in order to evaluate cardiomyocytes stained with uranyl acetate and lead citrate. Samples were visualized and photographed for microstructure alterations including the loss of cellular integrity and myofibril degradation. Prior to review, samples were coded without prior knowledge of their respective treatment regimens to eliminate observer bias.

F. APOPTOTIC PROTEIN ANALYSIS

An in-depth analysis of pro-apoptotic proteins (PARP, Caspase-3, and Bax) was measured using Western blot analyses. Protein isolation and quantification was performed from previously frozen murine heart tissue as previously described.[38] Approximately 30 µg of extracted protein underwent gel electrophoresis using 12% SDS-PAGE and transferred to a 0.45 mm PVDF membrane. Polyclonal antibodies to PARP, Caspase-3, and Bax (Cell Signaling) served as the primary antibody and were incubated with the separated protein samples overnight at 4°C. After washing, a secondary antibody probe was selected to label primary antibody which were then detected using the ECL Plus Chemiluminescent Detection Reagent (Western Lighting Plus-ECL, Amersham). Protein quantification was based on the

corresponding band intensity as measured by Quantity One image analysis software (BioRad Laboratories, Inc.).

G. STATISTICAL ANALYSIS

Statistical analysis of all echocardiographic parameters was expressed as mean ± SEM. An analysis of variance method (ANOVA) was used for the interpretation and comparison of repeated echocardiographic measurements taken over time. Statistically significant values were determined by a threshold p-value < 0.05.

For histological analysis, the Kruskal-Wallis Test was used to confirm the extent of cardiomyocyte remodelling using a non-parametric comparison of scores ranging from 1-4 with a score=4 representing severe damage. Finally, biochemistry results were determined by the two-tailed paired student's t-test. In both cases, a p-value of < 0.05 was considered significant.

RESULTS

A. MURINE ECHOCARDIOGRAPHY

At baseline, all murine echocardiographic parameters including heart rate (HR), LV cavity dimensions, and systolic function were comparable across the three assigned groups. Echocardiographic data from the saline control group remained unchanged from baseline to day 14. As compared to baseline, conventional echocardiographic indices demonstrated a significant decrease in LVEF in both BVZ and SNT experimental groups beginning at day 13. For BVZ-treated mice, this corresponded to an LVEF of 75 ± 2 % at baseline which was reduced to 48 ± 2 % by day 14 (Figure 4). Mice receiving SNT demonstrated a similar decline in the LVEF from 74 ± 2 % at baseline to 47 ± 3 % by day 14 (Figure 4). Both groups also demonstrated an associated increase in ventricular end-diastolic diameter (LVIDd). For BVZ-treated mice, this corresponded to an LVIDd of 3.1 ± 0.2 mm at baseline to an LVIDd of 3.9 ± 0.2 mm at day 14. Similarly, mice treated with SNT demonstrated an increase in LVIDd from 3.1 ± 0.2 mm at baseline to 3.9 ± 0.3 mm at day 14.

TDI parameters, including V_{ENDO} (normal value: >3 cm·s^{-1})[32] and SR (normal value: > 20 s^{-1}),[39] demonstrated normal baseline values for all mice. There was a significant reduction in V_{ENDO} and SR observed in mice treated with either BVZ or SNT as early as day 8 (Figure 5). For BVZ treated mice, V_{ENDO} initially decreased from a baseline value of 3.2 ± 0.1 cm·s^{-1} to 2.8 ± 0.1 cm·s^{-1} at day 8 and a final value of 1.7 ± 0.1 cm·s^{-1} at day 14. SR also declined over the course of the study in which a baseline value of 23 ± 2 s^{-1} decreased to 18 ± 3 s^{-1} at day 8 and a final day 14 value of 11 ± 2 s^{-1}. Similarly, in mice treated with SNT, baseline V_{ENDO} of 3.2 ± 0.2 cm·s^{-1} decreased to 2.7 ± 0.1 cm·s^{-1} at reaching a final value of 1.7 ± 0.1 cm·s^{-1} at day 14. SR decreased from 23 ± 2 s^{-1} at baseline to 19 ± 2 s^{-1} at day 8 and a day 14 value of 12 ± 2 s^{-1}.

B. BLOOD PRESSURE MONITORING

Mean arterial pressure (MAP) were within normal limits for all mice at baseline. Hemodynamic monitoring of the control group receiving 0.9% saline remained unchanged from baseline to day 14 (Figure 3). At day 14, mice treated with BVZ showed a significant increase in MAP from 90 ± 2 mmHg to 141 ± 5 mmHg ($p<0.05$). Similarly, the MAP increased from 89 ± 3 mmHg to 135 ± 4 mmHg ($p<0.05$) in mice treated with SNT.

C. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA analyses for all three treatment groups of mice had non-detectable levels of hsTnI and showed no significant difference in serum concentrations at baseline, day 7, and day 10 amongst groups. However, mice treated with either BVZ or SNT demonstrated elevations in hsTnI to 0.7 ± 0.3 ng/mL and 1.9 ± 0.9 ng/mL respectively at day 14 (Table 1).

D. HISTOLOGICAL ANALYSIS

Under transmission electron microscopy (TEM), approximately 16, 000 cardiac cells were scanned from three randomly derived blocks of tissue and evaluated by an independent reviewer. Samples revealed evidence of significant loss of cardiomyocyte cell integrity in wild-type C57Bl/6 mice treated with BVZ or SNT at the end of the two week period as compared to control (Figure 7). Specifically, myofibril degradation as well as dilatation of smooth endoplasmic reticulum and mitochondria organelles served as evidence of drug-induced cardiotoxicity.

E. APOPTOTIC PROTEIN ANALYSIS

In mice treated with BVZ or SNT, there was evidence of increased cardiomyocyte apoptosis as compared to controls. Specifically, Caspase-3, a pro-apoptotic protein, was found to be significantly elevated in BVZ- and SNT-treated mice as compared to controls (Figure 6). However, there was no significant evidence of increased PARP or Bax expression in mice treated with either BVZ or SNT.

DISCUSSION

In Canada, CRC and RCC collectively account for approximately 16% of all cancers diagnosed annually among men and women.[9] In these diseases, an improved understanding of the biochemical mechanisms of cancer and tumor cell progression has led to the development of novel drugs which act through inhibition of angiogenesis by targeting specific pro-angiogenic factors. Two such targeted therapies include the monoclonal antibody BVZ and the tyrosine kinase inhibitor SNT, respectively, used in the treatment of CRC and RCC. While these agents have proven more effective in the cancer setting, an unanticipated consequence of their utilization has been the development of cardiotoxicity. Our current study aimed to determine the potential application of cardiac biomarkers and tissue Doppler echocardiographic techniques in the early detection of cardiotoxicity. In an acute murine model, we have made a number of novel findings related to the detection and confirmation of BVZ or SNT mediated cardiotoxicity. First, novel echocardiographic indices including TDI and SR were convincingly able to detect early LV systolic dysfunction as compared to traditional LVEF assessment. Unfortunately, cardiac biomarkers assessment was an inadequate monitoring tool in early LV systolic dysfunction. Second, BVZ or SNT administration was associated with the development of systemic hypertension. Finally, investigation of cardiomyocyte histologic changes including cellular injury and myofibril disarray confirmed the onset of cardiac dysfunction in mice following BVZ or SNT treatment likely due to increased cell apoptosis.

In clinical practice, serial LVEF assessment remains the most common technique for routine cardiac function monitoring in cancer patients at risk of developing cardiotoxicity. However, LVEF impairment occurs only after considerable myocardial injury has occurred and fails to detect early alterations in LV systolic function. Specifically, a decreased LVEF represents significant cardiac injury following treatment with cardiotoxic chemotherapy, occurring only after compensatory myocardial contractile reserves have been exceeded.[19] In the setting of anthracycline-mediated cardiomyopathy, Mercuro et al. have previously validated that tissue Doppler echo indices and cardiac biomarkers accurately detected early preclinical changes of LV systolic function and were predictive of the subsequent risk for heart failure.[40] Thus, earlier indicators of myocardial injury using TDI echocardiographic techniques and cardiac biomarkers warrant further research in order to outline the full cardiac safety profile of anti-cancer agents, including BVZ and SNT.

In comparison to conventional echocardiographic techniques, TDI parameters have proven to be a reliable indicator of early alterations in myocardial functioning.[39] Using blood-flow

Doppler, ultrasound signals derived from myocardial tissue provides insight to the LV systolic function. In particular, V_{ENDO} which measures the peak torsional deformation during the ejection phase of the cardiac cycle can be measured using TDI.[21] SR, a measure of the rate of total deformation of the ventricular myocardium during a cardiac cycle, can also be acquired from TDI and has been regarded as the earliest and mildest sign of subclinical cardiotoxicity in the setting of chemotherapy-induced cardiotoxicity.[40] In both basic science and clinical studies, the utility of TDI-derived indices in the early detection of drug-induced cardiotoxicity has been strongly supported. Most notably, a murine study performed by Jassal et al. in 2009 demonstrated a significant decrease in both V_{ENDO} and SR as early as 24 hours following treatment with Doxorubicin, a well-established cardiotoxic chemotherapy.[34] Importantly, these TDI-derived parameters were an earlier indicator of cardiac dysfunction as compared to LVEF which only decreased a full four days after V_{ENDO} and SR reductions were noted. To further, clinical studies have corroborated the findings of animal models related to the early detection of Doxorubicin and Trastuzumab (DOX+TRZ) mediated cardiotoxicity. A prospective study completed by Fallah-Rad et al. from 2007-2009 demonstrated that SR values decreased as early as 3 months before significant LVEF declines were identified in breast cancer patients with anthracycline-mediated cardiomyopathy.[29] On the basis of this landmark study, multiple independent studies in the breast cancer setting have confirmed that TDI echo is an early sensitive marker of LV systolic dysfunction and hold prognostic value in determining the risk of more severe cardiac dysfunction.[21] However, there is an unequivocal paucity in information with regards to the specific application of TDI indices in the early detection of BVZ and SNT related cardiotoxicity.

In the specific setting of BVZ and SNT induced cardiotoxicity, the application of TDI in the detection of myocardial dysfunction is poorly explored. In a chronic murine model of BVZ-mediated cardiotoxicity, Chen et al. have previously determined a significant decrease in LVEF as compared to baseline at 3 month follow-up using small-animal MRI.[26] In our study, WT mice treated acutely with either BVZ or SNT demonstrated an expected decline in conventional echocardiographic indices as well as dilated LV cavity dimensions by day 13 (Figure 4). In contrast, novel TDI markers demonstrated significant findings as early as day 8 in the same subjects (Figure 5). Results from our study indicate the TDI assessment is a promising tool in detecting early subclinical cardiac impairment in this novel model. Thus, ASICS represents the first known murine study to validate the utility of TDI for the early detection of LV systolic dysfunction due to BVZ and SNT mediated cardiotoxicity. Furthermore, our research group has also generated preliminary clinical findings in collaboration with the Mayo Clinic, which has confirmed the novel finding that roughly 25% of patients with with CRC and RCC will develop severe LV systolic dysfunction due to treatment with either BVZ or SNT, and that TDI-derived indices were able to predict cardiotoxicity as early as one month into treatment.

Cardiac biomarkers have gained ample consideration as a viable method of detecting early myocardial injury following drug-induced cardiotoxicity. Cardiac troponins including TnI, are cardiomyocyte contractile proteins that are recognized as the most sensitive and specific method for the diagnosis of myocardial damage.[24] Investigations of cardiac biomarkers in the setting of BVZ and SNT related cardiotoxicity have been unsubstantial although preliminary animal models treated with BVZ conducted by Chen et al. have demonstrated significantly higher serum levels of TnI.[26] Similarly, a study performed by Cardinale et al. had previously confirmed that early serum detection of TnI was able to predict left ventricular dysfunction in breast cancer patients receiving high-dose chemotherapy.[4]

Clearly, limited knowledge exists in terms of the clinical value of cardiac biomarkers as an early marker of BVZ or SNT cardiotoxicity and remains an area of further inquiry. Elevations

in serum troponins are likely represent a continuum of cardiac injury ranging from reversible to irreversible, regardless of the mechanism. It has been suggested that troponins are released by surviving myocardium experiencing overload-induced remodelling following targeted therapy use.[24] Our acute murine model of drug-mediated cardiotoxicity demonstrated increases in hsTnI in both BVZ and SNT treated mice at the end of the fourteen day study. However, TnI measurements in both treatment groups showed no significant change from baseline at day 7 or day 10. Thus, our study demonstrated that targeted therapies of BVZ and SNT were related to a significant increase in hsTnI although the results were unable to validate the utility of cardiac biomarkers in the early detection of drug-induced cardiotoxicity.

In various studies, BVZ and SNT have both been associated with significant increases in blood pressure; although a defined pathophysiologic mechanism has yet to be firmly established. An existing model suggests that VEGF inhibition common to both targeted therapy agents leads to reduced endothelial NO production resulting in increased peripheral vascular resistance (PVR). Accordingly, *in vitro* studies performed on human endothelial cells demonstrated successful inhibition of endothelial NO synthase activity by targeting the VEGF pathway.[41] VEGF pathway inhibition subsequently led to a functional decrease in the arterioles and capillaries, resulting in an increased PVR and systolic blood pressure. Despite the substantial *in vitro* evidence of VEGF-related hypertension, *in vivo* animal models of BVZ and SNT hypertension have not been thoroughly explored. From a clinical perspective, systemic hypertension is a recognized side effect of BVZ and SNT. In CRC patients receiving adjuvant BVZ to a conventional chemotherapy regimen, Hurwitz et al demonstrated a 9% increased risk of developing severe hypertension as compared to chemotherapy alone.[5] Similarly, Rixe et al. determined a 23% overall risk of developing severe hypertension in patients receiving SNT for the treatment of RCC.[42] In our study, treatment with either BVZ or SNT resulted in an increase in MAP by nearly 50-55% as compared to baseline (Figure 3). As such, our laboratory findings are the first to demonstrate that in an *in vivo* murine model, treatment with either BVZ or SNT lead to systemic hypertension over a two week period. However, subsequent M-Mode analysis of LV cavity dimensions revealed no signs of left ventricular hypertrophy (LVH) commonly associated with chronic systemic hypertension. Still, it is more than likely that our acute model, on the scale of two weeks, was unable to maintain the prolonged hypertensive conditions necessary for hypertrophic cardiac remodelling to occur. In order to fully characterize the corollary of targeted therapy-related hemodynamic alteration, chronic administration of BVZ and SNT in a murine model is required.

At the microscopic level, cardiomyocyte alteration resulting from exposure to anti-angiogenic therapies, including BVZ and SNT, have been previously established. In particular, histological results from Chen et al. were able to determine decreased vascular density and signs of an ischemic cardiomyopathy following BVZ treatment.[26] Similarly, Chu et al. demonstrated cardiomyocyte degradation and mitochondrial swelling in a murine model of SNT mediated cardiomyopathy.[15] The current study revealed myofibril degradation, and dilatation of smooth endoplasmic reticulum and mitochondria organelles in cardiac tissue acquired from BVZ and SNT treated mice (Figure 7). Importantly, histologic examination of cardiac tissues revealed an absence of fibrotic scarring which may potentially identify BVZ and SNT related cardiac dysfunction as a reversible process.

Analysis of pro-apoptotic and anti-apoptotic gene products has previously been investigated in an effort to determine the pathophysiologic mechanism of cardiac dysfunction related to cardiotoxic agents. A previous animal trial performed by Jassal et al. confirmed that known cardiotoxic agents, DOX+TRZ, induced cardiac apoptosis with increases in PARP, Caspase-3, and Bax.[34] In an effort to elucidate the pathophysiology of BVZ and SNT cardiac

effects, our research group found a significant increase in the pro-apoptotic Caspase-3 in BVZ and SNT treatment arms as compared to controls although other apoptotic markers, PARP and Bax, revealed no difference (Figure 6). These findings are consistent with the current literature of TKI-mediated cardiotoxicity.[43] Isolated elevation in Caspase-3 would suggest that novel targeted therapies act through processes independent of DNA fragmentation-related apoptosis.[44] Accordingly, it is reasonable to assume that BVZ and SNT induced apoptosis is distinct from traditional Bax/Bcl mechanisms but does involve a caspase-dependent pathway. At a cellular level, the most plausible explanation for the biochemical results are likely related to the extraneous actions of BVZ and SNT beyond their intended anti-cancer targets. It is clear that additional insight in the underlying mechanisms of cardiomyocyte dysregulation and apoptosis are required in the setting of BVZ and SNT mediated cardiotoxicity.

All things considered, there were some limitations to our study that require attention. First, BVZ and SNT mediated cardiotoxicity was only evaluated in an acute murine model comprised of male WT mice over a period of fourteen days. Given that the clinical administration of these novel targeted therapies occurs over several months, translating the conclusions drawn from this study to the clinical setting may be challenging. As such, future studies will seek to establish a chronic model of drug-induced cardiac dysfunction over a time period of six weeks, in order to be more consistent with clinical practices. Second, a smaller number of parameters were only measured at baseline and at the end of the study, including non-invasive hemodynamic assessment and histologic analysis. Having identified alterations in cardiac functioning by day 8, significant findings of elevated MAP and cardiomyocyte changes may be used to support the conclusion of early subtle cardiotoxic effects related to BVZ and SNT. Finally, the exclusive use of hsTnI as a representative of cardiac biomarkers was a limitation of the study. Among patients treated with targeted therapies, numerous cardiac biomarkers including cardiac Troponin T (cTnT), CRP, and BNP have been identified as predictors of cardiac dysfunction. For future studies, alternative cardiac biomarkers will be considered as potential early markers for BVZ and SNT mediated cardiotoxicity.

CONCLUSION

Collectively, our study demonstrates that in an acute *in vivo* murine model, treatment with either BVZ or SNT is associated with systemic hypertension, LV systolic dysfunction, and cardiomyocyte injury. TDI-derived parameters were adequately able to detect ***early*** LV systolic dysfunction *prior* to alterations in conventional echocardiographic indices, representing the first time the modality has been assessed in terms of its predictive value of either BVZ or SNT mediated cardiotoxicity. Clinically, establishing subclinical markers of cardiac dysfunction in CRC patients receiving BVZ or RCC patients receiving SNT is already underway at the St. Boniface Research Centre collaboration with the Mayo Clinic. To further, the role of prophylactic cardioprotective agents in potentially reducing the onset of heart failure in these specific cancer patient populations remains to be seen.

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Figure 1: A schematic diagram illustrating the pathogenesis of BVZ and SNT mediated cardiotoxicity. Vascular endothelial growth factor, VEGF; Nitric oxide, NO; Phospholipase C-γ, PLC- γ; Protein kinase C, PKC; Mitogen-activated protein 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.

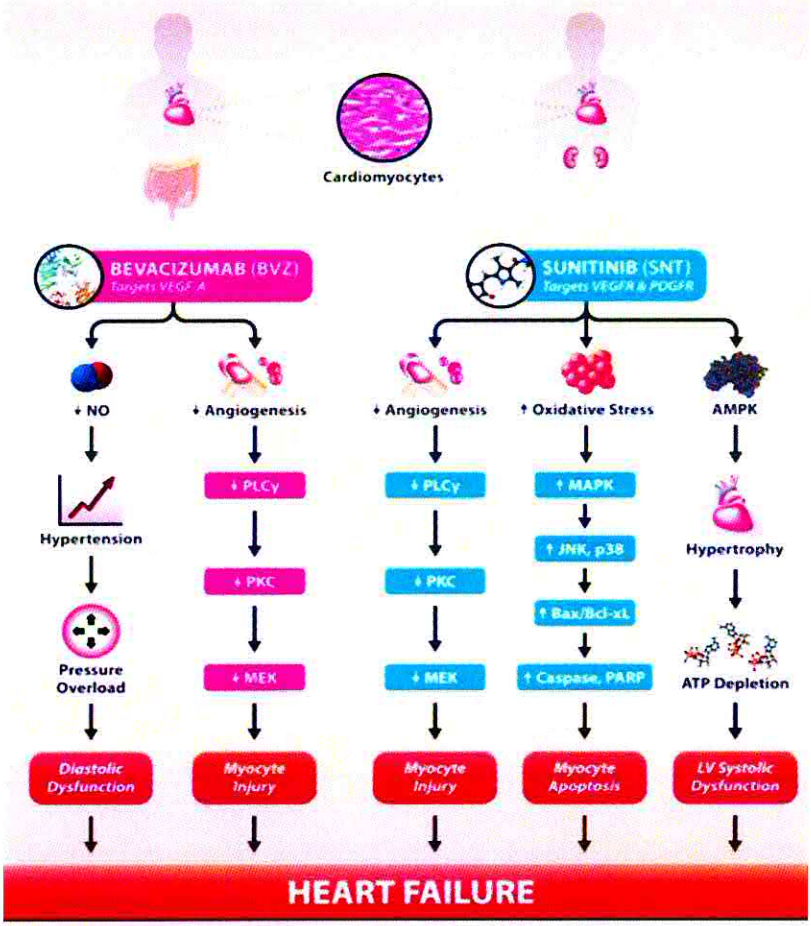


Figure 2: A total of 75 wild-type C57Bl/6 male mice were randomly assigned to one of the following drug regimens: i) 0.9% saline (i.p., n=5); ii) BVZ (10 mg/kg i.v., n=35); or iii) SNT (40 mg/kg/d orally, n=35). BVZ was administered once during the experimental protocol via parenteral injection. Sunitinib 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, day 10, and day 14. At the end of the experiment, the mice were euthanized for determination of histological and biochemical analyses.

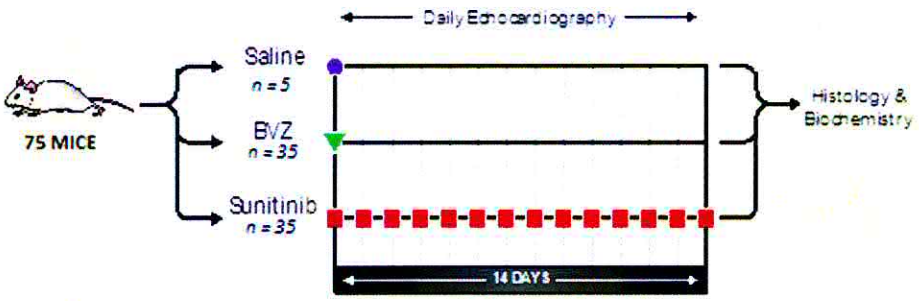


Figure 3: Mean arterial pressure (MAP) was measured non-invasively using a tail cuff method (CODA system, Kent Scientific, Torrington, CT) on conscious restrained C57Bl/6 mice treated with Saline, Bevacizumab (BVZ), Sunitinib (SNT) at baseline and Day 14. (*) significant for BVZ comparing Day 14 to baseline. ($p < 0.05$). (**) significant for SNT comparing day 14 to baseline. (#) significant comparing BVZ and Saline at day 14. (##) significant comparing SNT and Saline at day 14.

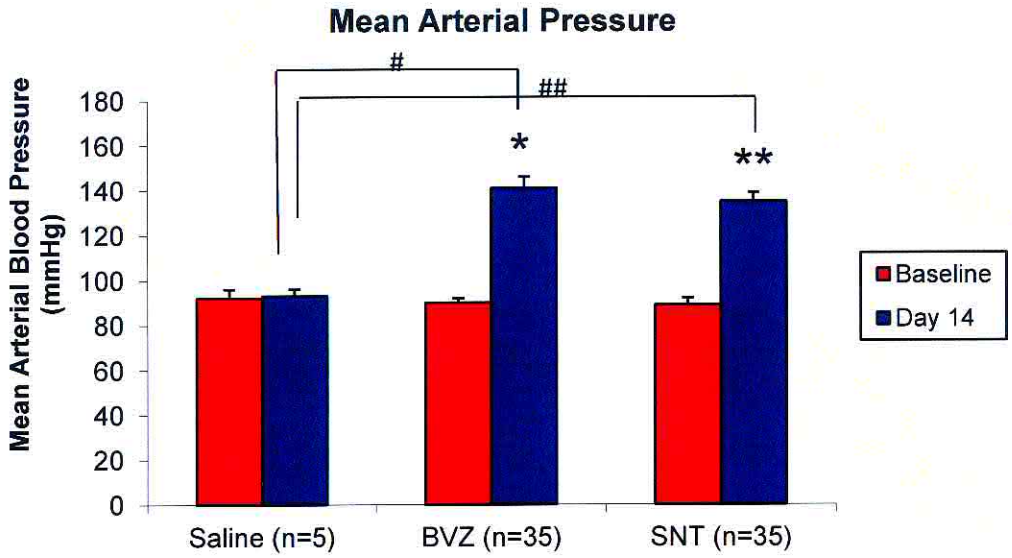


Figure 4: BVZ, Bevacizumab; SNT, Sunitinib. In the WT C57Bl/6 mice receiving 0.9% saline alone, there was no significant difference in LVEF at baseline and 14 days of follow-up. In mice treated with BVZ, the LVEF decreased from 76±3% at baseline to 52 ± 2% at day 13. Similarly, in mice treated with SNT, the LVEF decreased from 73±3% at baseline to 49 ± 1% at day 13.

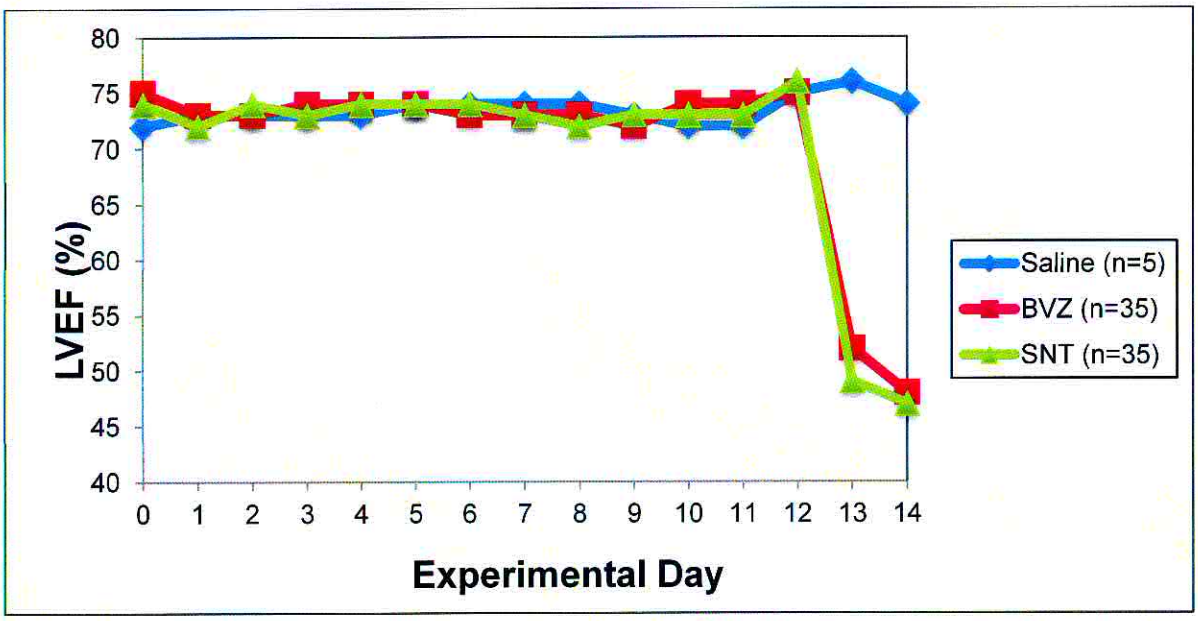
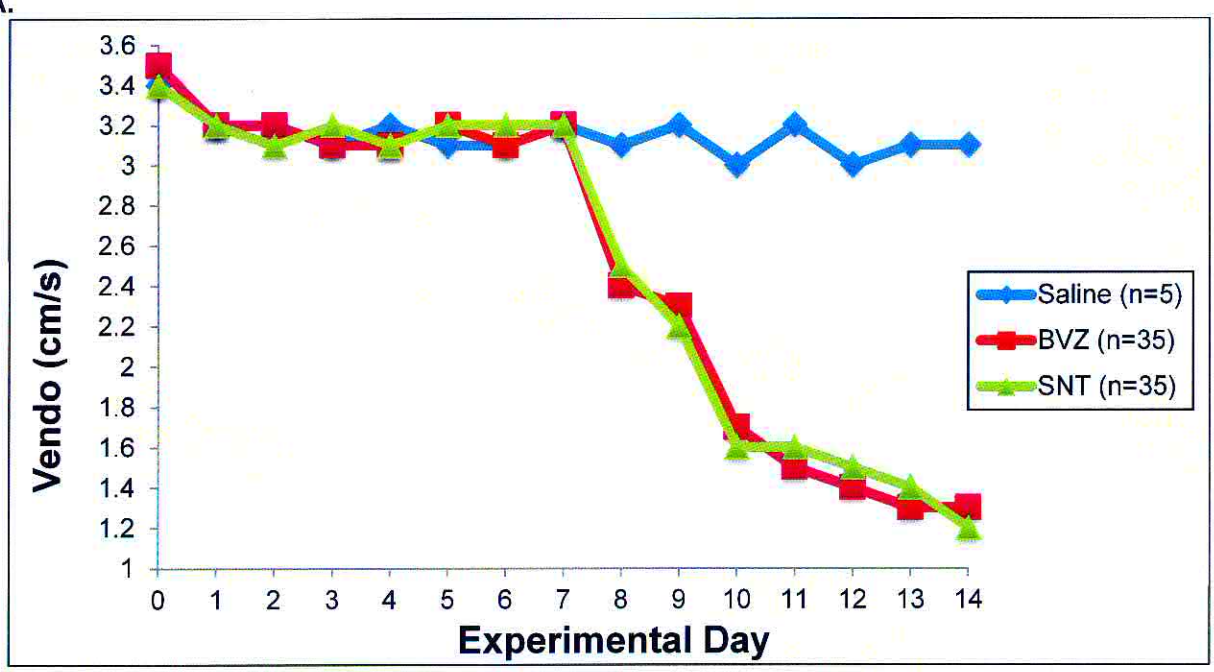


Figure 5: BVZ, Bevacizumab; SNT, Sunitinib; V_{ENDO}, endocardial velocity; SR, strain rate. In the WT C57Bl/6 mice receiving 0.9% saline alone, there was no significant difference in V_{ENDO} or SR at baseline and 14 days of follow-up. In mice treated with BVZ, the V_{ENDO} decreased from 3.5 ± 0.3 cm/s at baseline to 2.4 ± 0.1 cm/s at day 8. In mice treated with SNT, the V_{ENDO} decreased from 3.4 ± 0.2 cm/s at baseline to 2.5 ± 0.2 cm/s at day 8. The SR values in mice treated with either BVZ or SNT also demonstrated a significant decrease at day 8, confirming the detection of early subclinical LV systolic dysfunction.

A.



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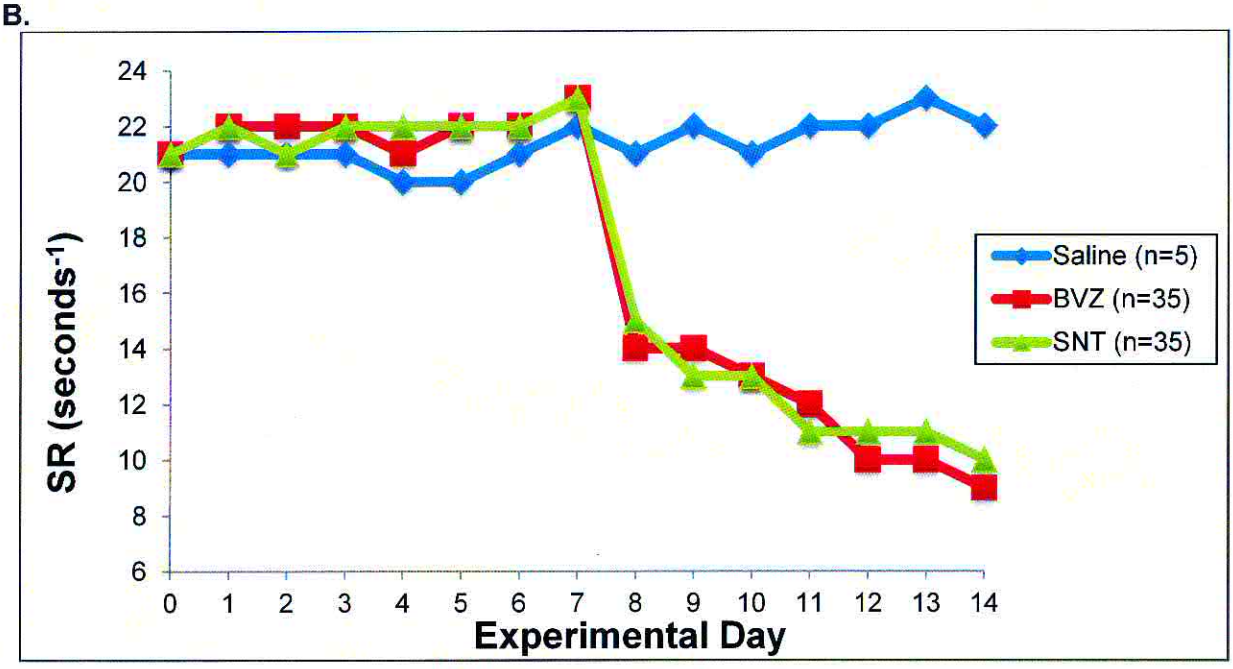

Figure 6: Caspase-3, pro-apoptotic gene product, was measured using Western blot analysis of previously frozen murine heart tissue from mice treated with Saline, Bevacizumab (BVZ), Sunitinib (SNT). (*) significant comparing BVZ to Saline. (#)significant comparing SNT to Saline.

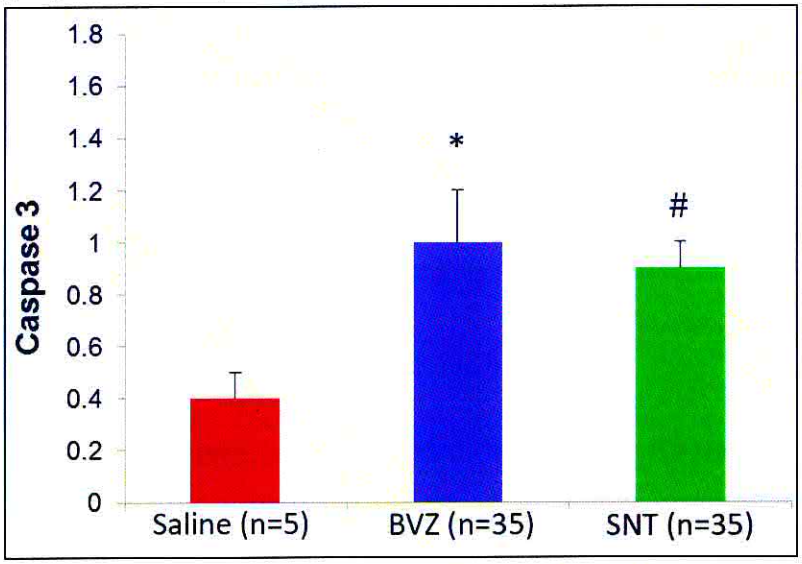

Figure 7: Electron microscopy of representative samples from WT mice treated with saline, BVZ or SNT. Original magnification x 5800 for all three images. WT, Wild type; BVZ, Bevacizumab; SNT, Sunitinib.

1. Control hearts with typical organelles and myofibrils
2. BVZ treated mouse demonstrating cells with loss of cellular integrity (arrows)
3. SNT treated mouse demonstrating cells with loss of cellular integrity (arrows)

Table 1: High sensitivity troponin I (hsTnI) concentration in serum measured by a mouse-specific ELISA at baseline, day 7, day 10 and day 14 (Life Diagnostics, Inc., West Chester, PA, US) in C57Bl/6 mice treated with either Saline, Bevacizumab (BVZ), Sunitinib (SNT). Values are reported in units of ng/ml and displayed as mean ± standard error of the mean (SEM). (*) significant comparing day 14 to baseline.

hsTnI Serum Concentrations				
Group	**Baseline**	**Day 7**	**Day 10**	**Day 14**
Saline (n=5)	0	0	0	0
BVZ (n= 35)	0	0	0	1.8 ± 0.3*
SNT (n = 35)	0	0	0	2.3 ± 0.4*