UNIVERSITY OF MANITOBA | MAX RADY COLLEGE OF MEDICINE
Rady Faculty of Health Sciences

Bachelor of Science in Medicine Degree Program
End of Term Final Report

Office of Graduate and Advanced Degrees
Education in Medicine Max Rady College of Medicine
Rady Faculty of Health Sciences
University of Manitoba

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Project Title: The Prophylactic Role of Renin-Angiotensin System Antagonism in the Prevention of Bevacizumab and Sunitinib Mediated Cardiotoxicity

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Summary (250 words max single spaced):

Background: The evolving field of Cardio-Oncology aims to improve the cardiovascular health of patients receiving anti-cancer therapy. Bevacizumab (BVZ) and Sunitinib (SNT) are novel targeted agents used in the treatment of colorectal (CRC) and renal cell cancer (RCC). Despite their effectiveness in prolonging overall survival, BVZ and SNT are associated with an increased risk of cardiotoxicity in up to 33% of patients. Although renin-angiotensin system (RAS) antagonists are commonly used after cardiac injury develops, their prophylactic role in the prevention of cardiotoxicity due to BVZ and SNT has yet to be investigated.
Objective: 1) To evaluate the prophylactic role of RAS antagonists in preventing the cardiotoxic side effects of BVZ and SNT in a chronic *in vivo* murine model; and 2) to elucidate potential mechanisms of the cardioprotective effects of RAS antagonists.
Methods: A total of 194 mice C57Bl/6 male mice received: i) 0.9% saline; ii) BVZ; or iii) SNT for 4 weeks. Within each arm, mice received prophylactic treatment with either water, Hydralazine, Aliskiren, Perindopril, or Valsartan via oral gavage for 28 days. Following serial echocardiographic and hemodynamic assessments for 4 weeks, hearts were collected for histological and biochemical analyses.
Results: In our murine model of BVZ and SNT induced cardiotoxicity, prophylactic administration of Aliskiren, Perindopril, and Valsartan: a) attenuated hypertension; b) mitigated adverse cardiac remodeling; c) preserved myofibril structural integrity; and d) decreased the extent of cardiac apoptosis.
Conclusion: In an *in vivo* murine model, prophylactic administration of RAS antagonists partially attenuated BVZ and SNT induced cardiotoxicity.

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Acknowledgments: I gratefully acknowledge the sole or partial funding support from the following sponsors;

H.T. Thorlakson Foundation
Dean, College of Medicine
Research Manitoba

Manitoba Medical Service Foundation (MMSF)
Vice-Dean, Research Rady FHS
Health Sciences Centre Research Foundation
Heart and Stroke Foundation

Sponsorship if different than above;

INTRODUCTION

Cancer is the leading cause of morbidity and mortality among Canadians, accounting for over 206,200 new diagnoses and 80,800 deaths projected for 2017.[1] Treatment has traditionally involved surgery, radiation, and chemotherapy. Over the last decade, an expanded understanding of molecular biology of cancer has contributed to the emergence of novel therapies which target tumour progression. Two widely utilized targeted agents include Bevacizumab (BVZ) and Sunitinib (SNT), which are used for treatment of colorectal (CRC) and renal cell cancers (RCC), respectively.[2–6] Although these anti-cancer medications are effective in improving overall survival, nearly 1 of every 3 patients receiving BVZ or SNT are at risk of cardiotoxicity.[7–10] The evolving field of Cardio-Oncology aims to improve the well-being of cancer patients who have pre-existing heart disease and/or develop cardiovascular complications due to cancer treatment.

CRC is the second most common type of cancer in Canada, with approximately 27,000 new diagnoses and 9,300 deaths in 2016.[1] Diet, lifestyle, advanced age, and genetic predisposition are the most prominent risk factors for developing CRC.[1,11] Treatment is individualized to each patient and may include tumour resection, radiation, chemotherapy, and the administration of targeted agents including BVZ. BVZ is a recombinant humanized monoclonal antibody of murine origin which antagonizes vascular endothelial growth factor (VEGF). VEGF is a critical determinant of the angiogenesis signalling cascade and is strongly associated with metastasis, making it a useful target for novel anti-cancer medications including BVZ.[12] By binding VEGF-A isoforms, BVZ prevents proliferation of vascular endothelial cells.[3,13,14] It is effective in inhibiting angiogenesis, slowing progression of tumours and prolonging patient survival.[13] BVZ is approved as first-line treatment of metastatic CRC in combination with 5-fluorouracil (5-FU) based chemotherapy.[3,14]

RCC is the tenth most common type of cancer in Canada, with approximately 6,400 new diagnoses and 1,850 deaths in 2016.[1] Prolonged exposure to petroleum, asbestos, analgesic use, smoking, and increased body mass index increases the risk of acquiring RCC.[15-18] The treatment for RCC is multifactorial including surgical nephrectomy or thermal ablation, followed by immunomodulatory therapy consisting of interferon-α or interleukin-2.[19] As advanced RCC is highly resistant to systemic chemotherapy,[19] novel biological agents have recently been added to the treatment regimen.[5] One such agent, Sunitinib (SNT), is an oral tyrosine kinase inhibitor targeting multiple receptor kinases, including VEGF receptors (VEGFR) 1-3, adenosine monophosphate-activated kinase (AMPK), and platelet-derived growth factor receptors (PDGFR) α and β.[4] By inhibiting the activity of these receptors, SNT slows neoplastic angiogenesis and tumour progression, and is effective as first-line therapy for metastatic RCC.[4–6]

Although BVZ and SNT provide effective treatment for patients with advanced CRC and RCC, respectively, these VEGF inhibitors pose a significant risk for development of cancer therapy related cardiac dysfunction (CTRCD).[20,21] Approximately a third of patients treated with BVZ may present with hypertension, arrhythmias, and left ventricular (LV) systolic dysfunction.[2,8,22,23] Furthermore, up to 65% of SNT-treated patients with advanced RCC may experience adverse cardiac sequelae, including hypertension and symptomatic heart failure.[4,9,10,24] With an increasing number of cancer survivors experiencing CTRCD, there is a need for research focused on the early detection and prevention of cardiotoxicity in this patient population.

A common mechanism for BVZ and SNT mediated cardiotoxicity involves the up-regulation of the renin-angiotensin system (RAS) (Figure 1). As both of these agents antagonize the VEGF signalling pathway, there is an increased production of cardiac Angiotensin-II (Ang-II), which stimulates activity of NADPH oxidase and oxidative stress (OS) production.[12,21,25] OS, in turn, promotes downstream activation of mitogen-activated protein kinases (MAPKs), including apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK), and p38.[9,24] Activation of the MAPK family results in increased expression of several pro-apoptotic genes (Bax, Caspases, and Poly (ADP-ribose) polymerase (PARP)) and decreased expression of anti-apoptotic genes (Bcl-xL).[21] Persistent stimulation of the RAS pathway leads to cardiac apoptosis and heart failure.[12,21,25] Thus, an essential component of first-line treatment for heart failure is inhibition of the RAS pathway.[26-29] This can occur at one of three levels: direct renin inhibitors (DRIs), angiotensin converting enzyme inhibitors (ACEIs), or angiotensin receptor blockers (ARBs).[26-31]

This study examines the question of whether RAS inhibitors can prevent cardiotoxicity in the setting of BVZ or SNT cancer treatment. Recent clinical research in various settings of CTRCD supports this hypothesis. The randomized control trial OVERCOME demonstrated that the combination treatment of prophylactic ACE inhibition (Enalapril) and beta blockade (Carvedilol) resulted in lower rates of heart failure and mortality in the setting of haematological malignancies.[32] Similarly, the recent MANTICORE study found cardioprotective effects of an ACEI (Perindopril) as well as of beta-blockade (Bisoprolol) in breast cancer patients treated with Trastuzumab (TRZ).[33] The clinical trial PRADA confirmed that prophylactic treatment with an ARB (Candesartan) was effective in preventing an overall decline in LVEF in breast cancer patients assigned to anthracycline therapy with or without TRZ.[34] Despite these encouraging findings, little is known about the potential cardioprotective role of RAS antagonists in the setting of BVZ and SNT mediated cardiotoxicity.

OBJECTIVE

The two main objectives of this basic science study are to: 1) Evaluate the prophylactic role of RAS antagonists in preventing the cardiotoxic side effects of BVZ and SNT in a chronic *in vivo* murine model; and 2) Elucidate potential mechanisms of the cardioprotective effects of RAS antagonists.

HYPOTHESIS

We hypothesize that BVZ or SNT induced cardiotoxicity will be mitigated by the prophylactic administration of RAS inhibitors. We further propose that the cardioprotective mechanism may involve a decrease in apoptotic markers with preservation of overall LV systolic function (Figure 1).

METHODS

A. CHRONIC MURINE MODEL OF BVZ OR SNT MEDIATED CARDIOMYOPATHY

All animal procedures were performed 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 University of Manitoba Protocol Review Committee.

For the *in vivo* chronic murine model of BVZ and SNT induced cardiotoxicity, a total of 194 wild-type C57Bl/6 male mice were randomly assigned to a 4 week regimen of either: i) 0.9% saline (weekly intraperitoneal injections (i.p.); n=39); ii) BVZ (10 mg/kg, weekly intravenous injections (i.v.) n=78); or iii) SNT (40 mg/kg, daily oral gavage; n=77) (Figure 2). In this chronic murine model, the dosage of BVZ and SNT stated above induce LV systolic dysfunction as validated by our group and others.[9,21,35]

Within each arm, mice were further randomized to receive *prophylactic* daily treatment with either water, Hydralazine (0.05 mg/mL), DRI (Aliskiren, 50 mg/kg), ACEI (Perindopril, 4 mg/kg), or ARB (Valsartan, 2 mg/kg) daily oral gavage for a total of 28 days. Prophylactic treatment with water, Hydralazine, or RAS blockade was administered prior to treatment with either BVZ or SNT on the same day. Aliskiren, Perindopril, and Valsartan were selected due to their increased water solubility. The various doses of these RAS antagonists listed above have been shown to provide sufficient RAS blockade in a murine setting.[36-39]

We predicted that the potential cardioprotective effects of the RAS antagonists will be independent of their anti-hypertensive abilities. To support this hypothesis, Hydralazine was used as a positive control. Hydralazine is an anti-hypertensive agent which does not affect the RAS pathway, and therefore, is not expected to significantly attenuate cardiotoxicity due to BVZ or SNT.

In vivo cardiac function using transthoracic echocardiography (TTE) and non-invasive hemodynamics were assessed at baseline and weekly for a total of 4 weeks. Upon the conclusion of the study, blood was collected from all mice via the internal jugular vein. All animals were then euthanized by i.p. injection of 150mg/kg pentobarbital buffered with 2% lidocaine, and hearts were harvested from the mediastina. Each heart was rinsed in 0.9% saline and halved. One half of each heart was preserved for further histological analysis and the other was flash frozen in liquid nitrogen for further biochemical analysis.

B. MURINE ECHOCARDIOGRAPHY

In vivo TTE was carried out in awake mice at baseline and weekly thereafter for 4 weeks using a 13-MHz probe (Vivid 7, GE Medical Systems, Milwaukee, Wi). Both the parasternal long axis (PLAX) and short axis (PSAX) views were imaged, as previously described.[40-43] The PLAX windows were used to trace LV end systolic and end diastolic volumes (LVESV and LVEDV), in order to calculate the LV ejection fraction (LVEF). The PSAX windows were used to derive the M-mode echocardiographic indices, including heart rate (HR), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), posterior wall thickness (PWT), and interventricular septal thickness (IVS). All images and calculations of LVEF and fractional shortening (FS) were processed offline using the EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US). Echocardiographic data collection and analysis were performed by observers (AS and DJ) blinded to the various treatment groups.

C. HEMODYNAMIC PARAMETERS

HR and BP were measured non-invasively using a tail cuff method (CODA system, Kent Scientific, Torrington, CT) on conscious, restrained mice at baseline and repeated weekly for the duration of the study, as previously described.[42,44]

D. HISTOLOGICAL ANALYSIS

For electron microscopy evaluations, harvested cardiac tissues were fixed for 3 hours with 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3 at room temperature. Next, samples were rinsed overnight at 4ºC in 0.1M phosphate buffer containing 5% sucrose. They were then post-fixed for 2 hours at room temperature with 1% osmium tetroxide in 0.1M phosphate buffer. Subsequently, tissues were dehydrated in solutions with increasing ethanol concentrations and embedded in Epon 812 according to standard protocol.[44] Finally, ultrathin sections were stained with uranyl acetate and lead citrate, and examined with the Philips CM12 electron microscope for cellular integrity, chiefly, mitochondria and contractile elements.[9,21]

E. WESTERN BLOTTING

A Western blot analysis was conducted to identify the expression levels of apoptotic markers, including PARP, Caspase 3, Bax, Bcl-xL, p38, and GAPDH. Frozen heart tissue was ground in liquid nitrogen, and protein was extracted by homogenization in the radioimmunoprecipitation (RIPA) buffer supplemented with protease and phosphatase inhibitors (Thermo Scientific). Samples were incubated on ice, centrifuged at 14,000 rpm at 4ºC for 15 min, and the supernatants were collected. 30 μg of extracted protein were separated by SDS-PAGE gel electrophoresis at 55mA for 1.5 hours and were transferred to 0.2 μm pore size PVDF membrane at 100V for 1 hour at 10ºC. Membranes were blocked in 5% skim milk powder or bovine serum albumin for 1 hour at room temperature. Polyclonal antibodies specific to PARP, Caspase 3, Bax, Bcl-xL, p38, and GAPDH (Cell Signaling) served as primary antibodies and probed the membranes overnight at 4ºC. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (BioRad) was then selected to label the primary antibody. Protein bands were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific) on CL-Xposure blue X-ray film (Thermo Scientific). Band intensities were quantified by densitometric analysis using QuantityOne software (Bio-Rad) and normalized to GAPDH as the loading control.

F. STATISTICAL ANALYSIS

All data were expressed as mean ± standard deviation (SD). For Western analysis, the data were expressed as mean ± standard error mean (SEM). For post hoc analysis, repeated measures of one-way analysis of variance (ANOVA) were used to evaluate for significance between independent factors. P values for main effects and interactions were recorded when appropriate. The Kruskal-Wallis and Mann-Whitney tests were used to compare degrees of histological tissue damage, which ranged from 1-4, with 4 representing severe damage. Hemodynamic, echocardiographic, and biochemical analyses were performed by ANOVA. Means were compared by ANOVA with Dunnet's post-hoc analysis. A p value of <0.05 was considered significant. The statistical software packages SPSS 15.0, SPSS version 24, and Graphpad Prism 5 were used to perform the analysis.

RESULTS

A. MURINE ECHOCARDIOGRAPHY

At baseline, echocardiographic parameters including HR, IVS, PWT, LVEDD, and LVEF were similar between all treatment groups. HR, IVS, and PWT remained within the normal range for all the treatment groups throughout the duration of the 28-day study.

In mice treated with BVZ, echocardiographic indices demonstrated a decrease in LVEF from 72±3% at baseline to 41±2% at week 4, confirming the development of LV systolic dysfunction (Figure 3). Similarly, prophylactic administration of Hydralazine resulted in a decrease in LVEF to 43±3%. Prophylactic administration of Aliskiren, Perindopril, or Valsartan was cardioprotective with LVEF values of 57±2%, 50±2%, and 51±3% at day 28, respectively ($p<0.05$).

Comparable results were observed in mice treated with SNT. At day 28, LVEF decreased from 73±4% at baseline to 34±3% in the treatment arm (Figure 3). Mice treated prophylactically with Hydralazine demonstrated a similar decrease in LVEF to 33±4%. Prophylactic administration of Aliskiren, Perindopril, or Valsartan was partially cardioprotective with LVEF values of 54±2%, 45±2%, and 44±3% at week 4, respectively ($p<0.05$).

C. HEMODYNAMICS

The mean arterial blood pressure (MAP) of mice treated with saline was normal at baseline and week 4 (Figure 4).

In mice treated with BVZ, MAP rose to 136±4 mmHg at week 4. Prophylactic treatment with Hydralazine mitigated the rise of MAP, which measured at 124±3 mmHg by the end of the study. Treatment with Aliskiren, Perindopril, or Valsartan similarly attenuated the rise in blood pressure due to BVZ, resulting in MAP values of 124±4 mmHg, 125±5 mmHg, or 123±6 mmHg, respectively.

Analogous results were obtained in the SNT treatment arm. In mice treated with SNT alone, MAP increased to 138±3 mmHg on day 28. Prophylactic treatment with Hydralazine mitigated the rise of MAP, which measured at 123±3 mmHg by the end of the study. Prophylactic treatment with Aliskiren, Perindopril, or Valsartan attenuated the rise in blood pressure due to SNT, resulting in MAP values of 124±3 mmHg, 125±4 mmHg, or 124±5 mmHg, respectively.

E. HISTOLOGICAL ANALYSIS

Cellular integrity of the LV cardiomyocytes was preserved in mice treated with saline throughout the study (Figures 5-6). Electron microscopy depicted myofibril degradation and sarcomere disarray in cardiac tissue of mice treated with BVZ or SNT alone, representing severe cardiomyocyte damage.

Addition of Hydralazine to the treatment regimen resulted in similar myofibril degradation in BVZ treated mice. Conversely, in the SNT treatment arm, Hydralazine conferred a cardioprotective benefit, leading to less structural degeneration ($p=0.0006$).

Prophylactic administration of Aliskiren and Perindopril partially attenuated injury in both the BVZ treatment arm ($p=0.02$ and $p=0.003$, respectively), and in the SNT treatment arm ($p=0.0001$ and $p<0.0001$, respectively). Prophylactic administration with Valsartan led to partial cardiomyocyte preservation in the SNT treated animals ($p<0.0001$), but not in mice receiving BVZ ($p=0.08$).

F. WESTERN BLOTTING

Cleaved PARP protein is involved in programmed cell death; a rise in its levels signifies increased apoptosis. At the end of the study, a 2.1-fold increase in cleaved PARP protein

expression was observed in mice treated with BVZ alone, compared to mice receiving saline (Figure 7). Addition of Hydralazine also led to a 2.0-fold increase in this pro-apoptotic marker. Prophylactic treatment with Aliskiren, Perindopril, and Valsartan was significantly cardioprotective, resulting in only a 1.5-fold increase of cleaved PARP protein expression.

Similar results were shown in the SNT treatment arm. Cleaved PARP protein levels rose 2.3-fold in mice treated with SNT alone. Addition of Hydralazine to SNT was not significantly cardioprotective, as cleaved PARP protein concentration remained elevated 2.2-fold (Figure 7). Aliskiren, Perindopril and Valsartan mitigated the rise in the levels of this protein, allowing for only a 1.4-fold increase.

DISCUSSION

Cardio-Oncology is a developing interdisciplinary field tasked with detection, treatment, and prevention of CTRCD. Despite their effectiveness in treating metastatic disease, new targeted therapies, including the VEGF inhibitors BVZ and SNT, are associated with an increased risk of cardiotoxicity. Our novel study aimed to evaluate potential cardioprotective benefits of prophylactic blockade of the RAS in the setting of BVZ and SNT induced cardiomyopathy. We demonstrated that in a chronic *in vivo* murine model of BVZ and SNT mediated cardiotoxicity, RAS antagonists prevented adverse cardiovascular remodelling. Specifically, the prophylactic administration of RAS antagonists: i) attenuated the rise in MAP; ii) mitigated adverse cardiac remodelling; iii) preserved myofibril structural integrity; and iv) decreased the extent of cardiac apoptosis.

Hypertension is the most frequent and well-recognised manifestation of CTRCD in clinical studies of BVZ and SNT mediated cardiomyopathy.[45] The proposed mechanism of BVZ and SNT induced hypertension includes decreased nitric oxide (NO) production, capillary rarefaction, increased endothelin-1 synthesis, and up-regulation of the RAS pathway.[12,46-49] Animal studies confirm development of this side effect in the setting of BVZ and/or SNT administration. Belcik and colleagues recently investigated the effects of administration of a VEGF-A monoclonal antibody analogous to BVZ, in a chronic murine model.[50] Their report documented a significant rise in systolic blood pressure of 17 mmHg during the first week of antibody administration.[50] The authors attributed this change to upregulated Ang-II levels, with resultant increased afterload and LV remodeling.[50] Our group's recent evaluation of BVZ and SNT mediated cardiotoxicity in an acute murine model corroborates these important findings.[21] On day 7 of the study, mice in both the BVZ (10mg/kg) and SNT treatment arms (40mg/kg/d) demonstrated an increase in MAP by 30 mmHg and 26 mmHg, respectively.[21] In our present report using a chronic model, we further confirmed the development of systemic hypertension in BVZ and SNT treated mice. On day 28 of our study, we observed a significant rise in MAP of up to 20 mmHg, as compared to baseline. All of the murine studies above confirm the development of systemic hypertension in mice receiving VEGF-inhibitors.

Little is known about the effect of RAS antagonists on mitigating the development of BVZ and SNT induced hypertension. In the above-mentioned study by Belcik *et al*., treatment with ACEI (Ramipril, 5 mg/kg) fully prevented the rise in systemic blood pressure in mice treated with the monoclonal antibody equivalent to BVZ.[50] Similarly, our present study concluded that administration of a vasodilator or RAS inhibitors prevented the development of BVZ and SNT mediated hypertension. All four prophylactic agents, including Hydralazine, Aliskiren, Perindopril

or Valsartan were effective in suppressing BVZ and SNT mediated hypertension throughout the duration of the study.

Although the cardioprotective role of RAS antagonists has not yet been explored in the clinical setting of CRC and RCC, two recently completed investigations in the breast cancer population show promising results. In the MANTICORE trial, prophylactic administration of an ACEI (Perindopril, 2mg daily) led to significant attenuation of systemic hypertension in patients receiving Trastuzumab (TRZ).[33] The PRADA trial similarly demonstrated that the combination of an ARB (Candesartan) and a beta-blocker (Metoprolol) prevented the development of chemotherapy mediated hypertension.[34] Our current report confirmed for the first time that RAS inhibitors effectively prevented the rise in MAP in the setting of chronic murine BVZ and SNT induced cardiomyopathy. Further investigation of the prophylactic antihypertensive role of these agents is warranted in the clinical setting of CRC and RCC.

Serial LVEF assessment with transthoracic echocardiography (TTE) remains the principal diagnostic tool in the determination of CTRCD. Expert guidelines define CTRCD as a decline of LVEF greater than 10% to a value below 53%.[51] Both acute and chronic animal studies have investigated the utility of LVEF imaging in detecting CTRCD associated with BVZ and SNT treatment. In a study by Chen et al. using mice inoculated with human breast cancer and CRC cells, long-term treatment with BVZ (10 mg/kg) and 5-FU (15 mg/kg) resulted in a progressive decline in LVEF of up to 15%.[35] Furthermore, a 3 week study by Chintalgattu et al. documented a decreased in LVEF of up to 28% in SNT treated mice (40mg/kg/d).[24] In a previous acute murine model of BVZ (10mg/kg) and SNT (40mg/kg/d) induced cardiomyopathy, our group demonstrated a decline in LVEF values of approximately 25% in both groups as compared to baseline.[21] The present study extends these findings. In our chronic murine model, LVEF values decreased by approximately 30% and 40% in BVZ and SNT treated mice, respectively.

Prophylactic treatment with Hydralazine (H) did not preserve LVEF in our model. At the end of the study, we continued to observe a 30% and 40% decrease in LVEF in BVZ+H and SNT+H treatment groups, respectively. Although Hydralazine facilitated a decrease in MAP, it was not cardioprotective in an *in* vivo model of BVZ and SNT induced cardiac injury. In contrast, administration of RAS antagonists was cardioprotective in preventing LV systolic dysfunction. As both RAS antagonists and Hydralazine possess anti-hypertensive qualities, but only the former prevented adverse cardiovascular remodeling, we conclude that the cardioprotective effects of the RAS antagonists are independent of their blood pressure lowering effects. This finding further substantiates the pivotal role the RAS pathway plays in the development of CTRCD.

Of interest, we observed a trend wherein the DRI (Aliskiren) provided a greater preservation of LVEF, compared to the other two RAS antagonists, the ACEI (Perindopril) and the ARB (Valsartan). This observation corroborates an analogous finding in a recent chronic murine model of DOX+TRZ mediated cardiotoxicity that demonstrated that Aliskiren, Perindopril, and Valsartan were partially cardioprotective, with Aliskiren achieving the greatest preservation of LVEF.[52] We attribute this trend to the fact that Aliskiren directly inhibits renin (Figure 1), thus interfering with the earliest, rate limiting step of the RAS cascade. As such, it effectively suppresses angiotensin I and II, as well as the downstream aldosterone production, confering the greatest inhibition on the RAS pathway.[53]

Clinical studies further substantiate the utility of LVEF imaging in evaluating the cardioprotective effects of the RAS antagonists. The randomized clinical trial OVERCOME evaluated the prophylactic benefits of a combination treatment of ACEI (Enalapril) and a beta blocker (Carvedilol) in patients with haematological cancers receiving chemotherapy over 6 months.[32] Prophylactic treatment resulted in lower rates of LV systolic dysfunction, qualified as heart failure or LVEF <45%, or death. In the PRADA study, an ARB (Candesartan) attenuated decline in LVEF in breast cancer patients receiving anthracycline, taxanes, or TRZ.[34] Whether prophylactic administration of RAS antagonists may be cardioprotective in the setting of either CRC or RCC warrants further study.

Histological analysis is useful in revealing cardiomyocyte cellular changes following treatment with targeted agents including BVZ and SNT. In the 12-day model by Chu et al. of mice treated with SNT (40 mg/kg), electron microscopy demonstrated loss of cardiomyocyte integrity, including swelling of mitochondria and disrupted cristae.[9] Similar results were previously demonstrated by our group in an acute murine model of BVZ and SNT mediated cardiomyopathy, including loss of myofibril architecture and disruption of sarcomeres at day 14.[21] Our present chronic model extends these histological findings. Administration of both BVZ and SNT contributed to severe damage to cardiomyocyte architecture, including loss of sarcomere structure and myofibril disarray. Additionally, in both BVZ and SNT treatment groups, electron microscopy analysis detected an increase in mitochondria size and alterations in the appearance of cristae. Structural damage was more pronounced in SNT treated mice as compared to BVZ treated animals, corresponding with our previous echocardiographic observation of more profound loss of systolic function in the SNT treatment group.

In the BVZ treatment arm, addition of Hydralazine did not prevent myofibril disintegration. This contrasted with the SNT group, where Hydralazine administration was partially cardioprotective. The mechanism of this partial structural preservation by Hydralazine is not yet elucidated. We hypothesize that a longer duration of prophylaxis with Hydralazine may be needed in order for this antihypertensive agent to be cardioprotective in the setting of BVZ treatment.

Conversely, RAS inhibition, including Aliskiren and Perindopril, was cardioprotective in both BVZ and SNT treatment groups, revealing relative preservation of structural integrity of sarcomeres and myofibrils. This histological observation concurs with our echocardiographic, hemodynamic, and biochemical investigations, confirming cardioprotective benefits of RAS antagonists. The exception to these results was Valsartan, which was not deemed cardioprotective on histological analysis in our BVZ treatment group, as it failed to significantly attenuate loss of myofibril integrity on electron microscopy. This finding contradicted other study parameters, including echocardiography, hemodynamics, and biochemical analyses, in which Valsartan demonstrated cardioprotective properties. In the SNT treatment group, Valsartan provided cardioprotective benefits similar to other RAS antagonists, preserving cardiomyocyte integrity on electron microscopy. We anticipate that in future studies, increasing sample size in the BVZ treatment group may be necessary to reach statistical significance in the histological analysis.

Apoptosis is a process of programmed cell death that has been associated with BVZ and SNT mediated cardiotoxicity. Intrinsic and extrinsic biochemical stimuli that lead to this highly regulated process include hypoxia, free radicals, toxins, and chemotherapy agents.[54-56] As a result of these initiating events, members of the Bcl-2 protein family stimulate mitochondria to

release cytochrome *c* into the cytosol.[54] Bcl-2 protein family includes pro-apoptotic protein, Bax, which regulates whether a cell goes through self-destruction, and an anti-apoptotic protein Bcl-xL, which is responsible for terminating cell death.[54] Cytochrome *c* activates a large quaternary protein, apoptosome, which in turn, cleaves and activates pro-caspase 9 into its active form. The latter facilitates PARP proteolysis which is directly implicated in apoptosis.[54,55]

In an *in vivo* study of neonatal rats treated with SNT (1 μM), increased mitochondrial release of cytochrome *c* resulted in activation of pro-caspase 9 and up-regulation of cardiomyocyte apoptosis.[9] Conversely, Hasinoff et al. reported on an *in vivo* murine model of SNT mediated cardiotoxicity (up to 10 μM) where Bax levels remained unchanged, suggesting unchanged rates of cytochrome *c* release, and absence of cardiomyocyte apoptosis.[57] In the latter study, however, Hasinoff et al. reported higher levels of caspase-3 and caspase-7, proteins cleaved by activated caspase 9, indicating a possible alternate mechanism of cytochrome *c* release.[57] Furthermore, our group previously confirmed the role of apoptotic markers in detecting cardiotoxicity associated with targeted agents.[21] In our recent acute murine study of BVZ (10mg/kg) and SNT (40mg/kg) treated mice, caspase-3 levels were significantly increased, contrasting with unchanged Bax and PARP levels.[21] The above findings by Hasinoff's and our group suggest that Bax and Bcl-xL might not control the release of cytochrome *c*, warranting further research into this mechanism.

Our present chronic murine model revealed significantly elevated expression of cleaved PARP in both BVZ and SNT treatment groups at week 4, indicating increased apoptosis. Similarly, prophylactic administration of Hydralazine to either BVZ or SNT group did not attenuate apoptotic changes, as expression of cleaved PARP stayed significantly elevated. The latter finding, combined with our echocardiographic and histological results, confirmed that Hydralazine failed to provide cardioprotection, despite its anti-hypertensive effect. On the other hand, prophylactic addition of RAS antagonists to either BVZ or SNT treatment groups was deemed cardioprotective, as it mitigated the rise in cleaved PARP expression at Day 28. This observation further validates the utility of the RAS antagonists in preventing BVZ or SNT mediated cardiotoxicity.

A number of limitations exist in our present study. First, only male mice were used in this investigation of BVZ and SNT induced cardiac injury. Although in Canada, males are more frequently affected by CRC and RCC, cardiotoxicity related to BVZ and SNT treatment affects both sexes.[1] In the future, it would be useful to evaluate potential cardioprotective utility of RAS antagonists in a chronic model of BVZ and SNT treated female mice. Additionally, we did not assess the effectiveness of BVZ and SNT in tumour suppression when combined with RAS antagonists. In animal studies, ACEIs and ARBs demonstrate an ability to aid tumour inhibition.[58] Clinical research similarly demonstrates longer progression free survival times in patients treated with ACEIs and ARBs compared to those receiving VEGF inhibitors only.[59,60] It will be beneficial to further investigate the influence of RAS blockade on the cytotoxic abilities of BVZ and SNT in the cancer setting. This will further aid the goal of our Cardio-Oncology program to develop agents which offer cardioprotection while maximally potentiating the anti-cancer efficacy of BVZ and SNT. Finally, our current study employed a 4 week *in vivo* murine model to investigate the role of RAS antagonists in attenuating CTRCD. In clinical scenarios, however, RCC treatment including SNT is administered over 4 cycles of 6 weeks each, totalling 6 months.[6,61] CRC treatment, including BVZ, is similarly chronic. It would thus be valuable to

examine the potential cardioprotection of RAS antagonists over a treatment duration that more closely approximates clinical use.

In the future, we would like to test other vehicles that offer potential cardiac structural and functional preservation. In particular, spironolactone, statins, and β-blockers might afford cardioprotective qualities in the setting of BVZ and SNT mediated cardiotoxicity.

CONCLUSION

In a chronic *in vivo* murine model, RAS antagonists including Aliskiren, Perindopril, and Valsartan were able to attenuate cardiac injury induced by BVZ and SNT therapy. Future clinical studies are warranted to implement these basic science findings into the clinical world, with the goal of ultimately improving the well-being of advanced CRC and RCC patients.

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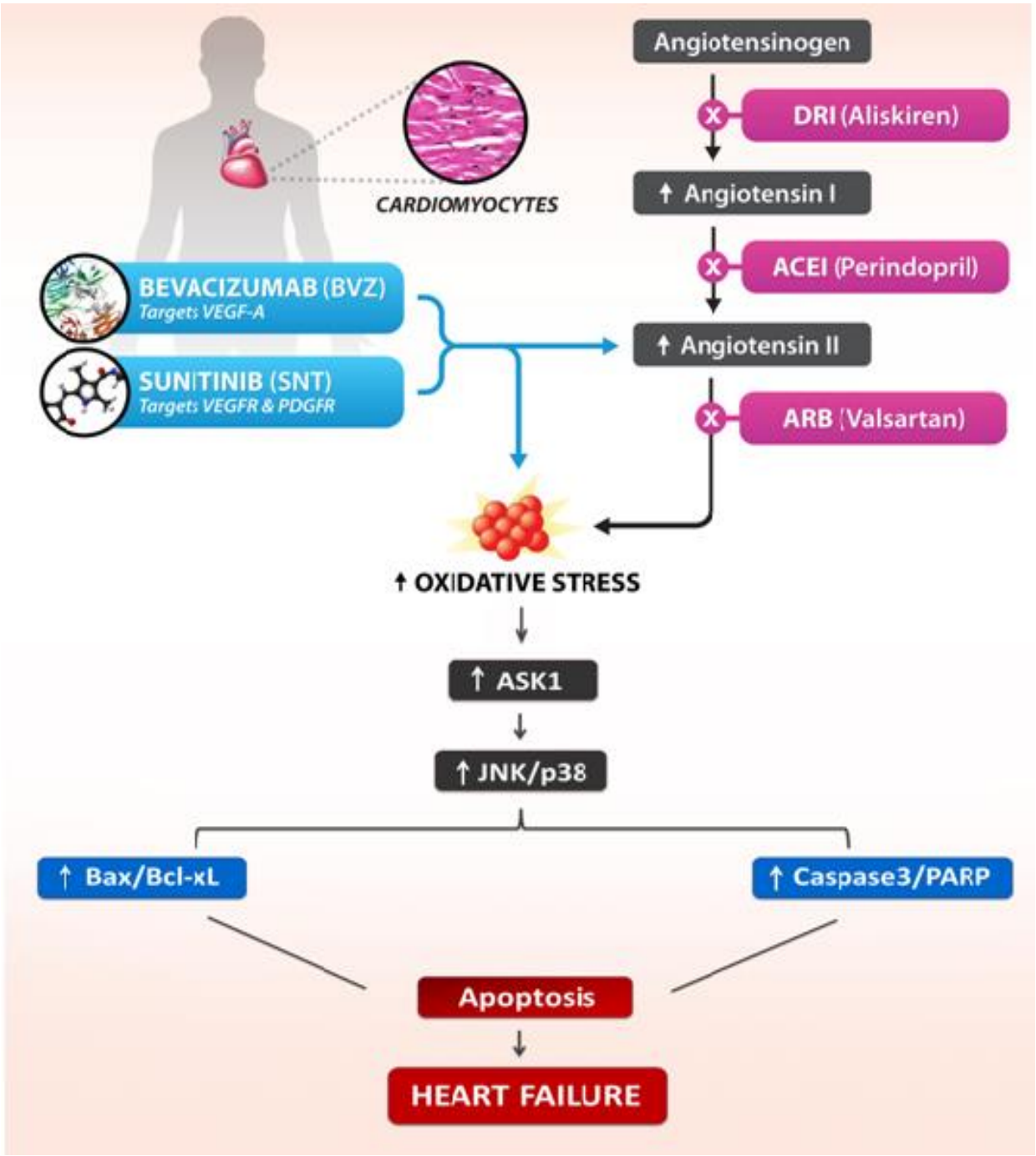

Figure 1: Pathogenesis of BVZ and SNT mediated cardiotoxicity and the RAS pathway. In experimental models of cardiac injury due to BVZ or SNT, up-regulation of RAS results in an increase in oxidative stress, leading to downstream activation of the MAPKs (ASK1, JNK and ps8), increased expression of Caspase-3, PARP, and Bax, decreased levels of Bcl-xL, and ultimately, increased cardiac apoptosis and heart failure. This study proposes that prophylactic administration of RAS antagonists Aliskiren, Perindopril, or Valsartan, will mitigate BVZ- or SNT-induced cardiotoxicity, through decreased OS and downstream activation of the MAPK cascade, diminished cardiac apoptosis and preservation of overall cardiac function.

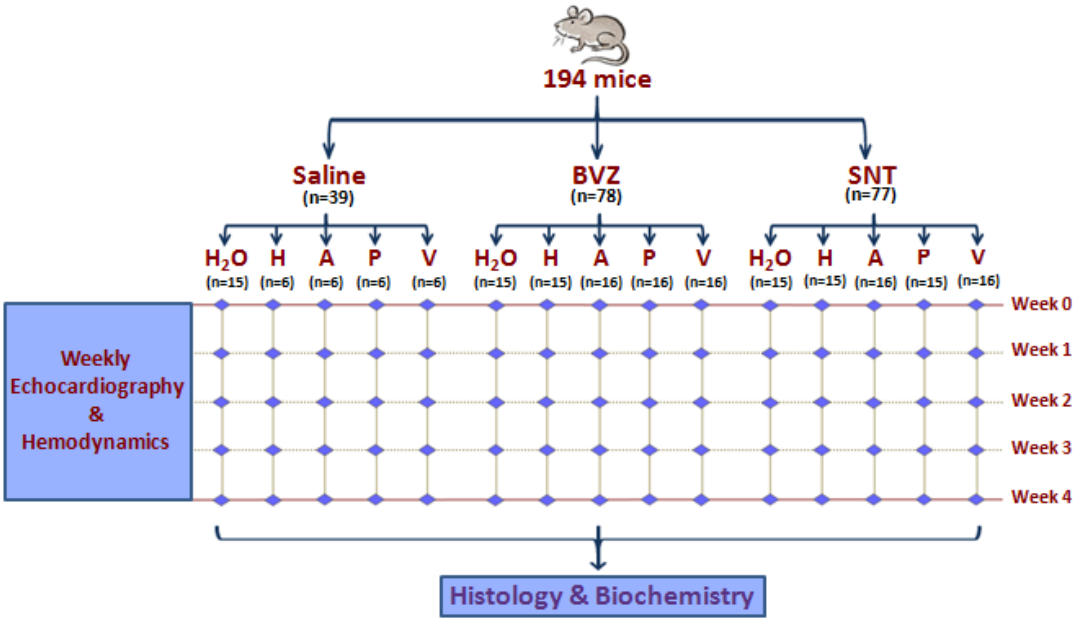

Figure 2: Experimental Protocol.
A total of 194 C57Bl/6 male mice were randomly assigned to either: i) 0.9% saline (i.p. weekly; n=39); ii) BVZ (10 mg/kg i.v. weekly for 4 weeks; n=78); or iii) SNT (40 mg/kg/day via oral gavage for 4 weeks; n=77). Within each arm, mice were further randomized to receive prophylactic treatment with either water (daily), Hydralazine (0.05 mg/ml/daily), DRI (Aliskiren 50 mg/kg/daily), ACEI (Perindopril 4 mg/kg/daily), or ARB (Valsartan 2 mg/kg/daily) via oral gavage for a total of 28 days. The mice underwent weekly echocardiographic and hemodynamic assessments at baseline and weekly thereafter. Upon the conclusion of the study, hearts were harvested for histological and biochemical analyses. BVZ, Bevacizumab; SNT, Sunitinib; H_2O, water; H, Hydralazine; A, Aliskiren; P, Perindopril; V, Valsartan.

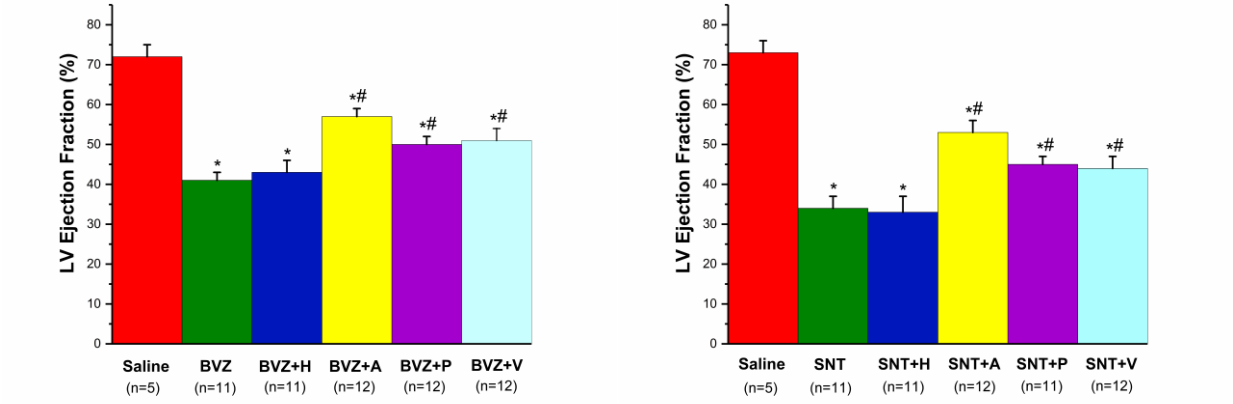

Figure 3: Conventional LVEF values in C57Bl/6 mice treated with either 0.9% saline, BVZ, SNT, and/or prophylactic administration of Hydralazine or RAS antagonists at day 28.
The results are reported as mean ± SD. *p<0.05 as compared to Saline. #p<0.05 as compared to BVZ or SNT alone. LV, left ventricular; BVZ, Bevacizumab; SNT, Sunitinib; RAS, renin-angiotensin system; H, Hydralazine; A, Aliskiren; P, Perindopril; V, Valsartan.

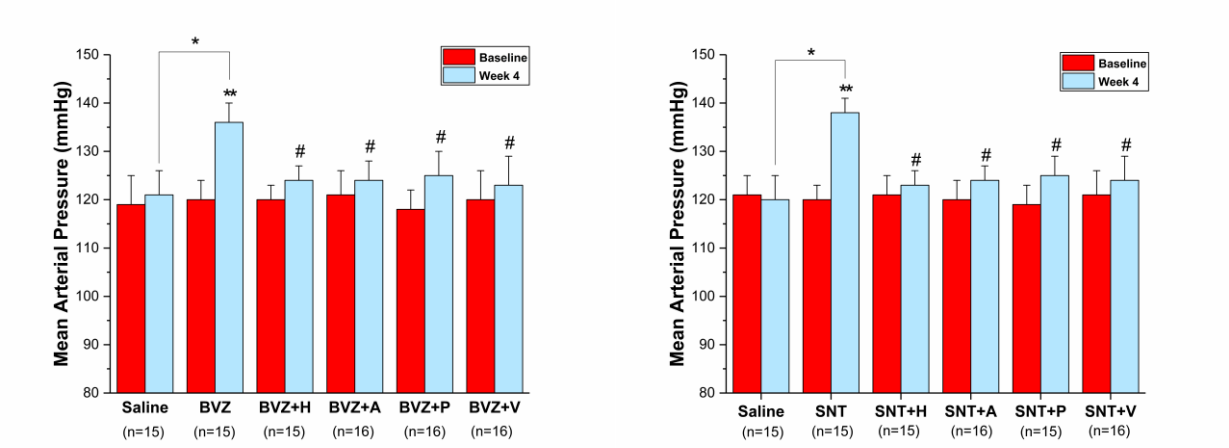

Figure 4: Mean arterial pressure in C57Bl/6 mice treated with either 0.9% saline, BVZ, SNT, and/or prophylactic administration of Hydralazine or RAS antagonists at baseline and week 4. The results are reported as mean ± SD. *p<0.05 between BVZ or SNT at week 4 as compared to Saline. **p<0.05 between BVZ or SNT at week 4 as compared to BVZ or SNT baseline. #p<0.05 as compared to BVZ or SNT at week 4. BVZ, Bevacizumab; SNT, Sunitinib; RAS, renin-angiotensin system; H, Hydralazine; A, Aliskiren; P, Perindopril; V, Valsartan.

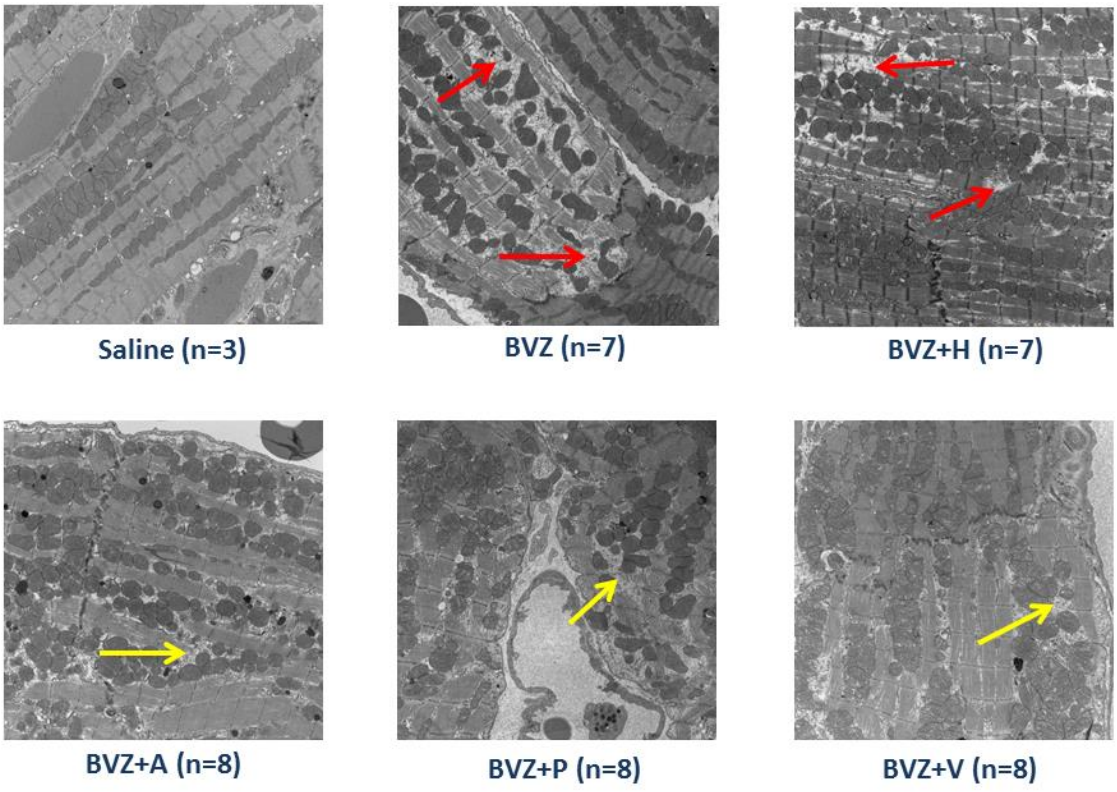

Figure 5: Representative electron microscopy images of heart samples from mice in the BVZ treatment arm. Images were taken at 5,800x magnification. BVZ alone or in combination with H resulted in severe myofibril disruption (red arrows). The prophylactic administration of RAS antagonists was partially cardioprotective (yellow arrows). BVZ, Bevacizumab; RAS, renin-angiotensin system; H, Hydralazine; A, Aliskiren; P, Perindopril; V, Valsartan.

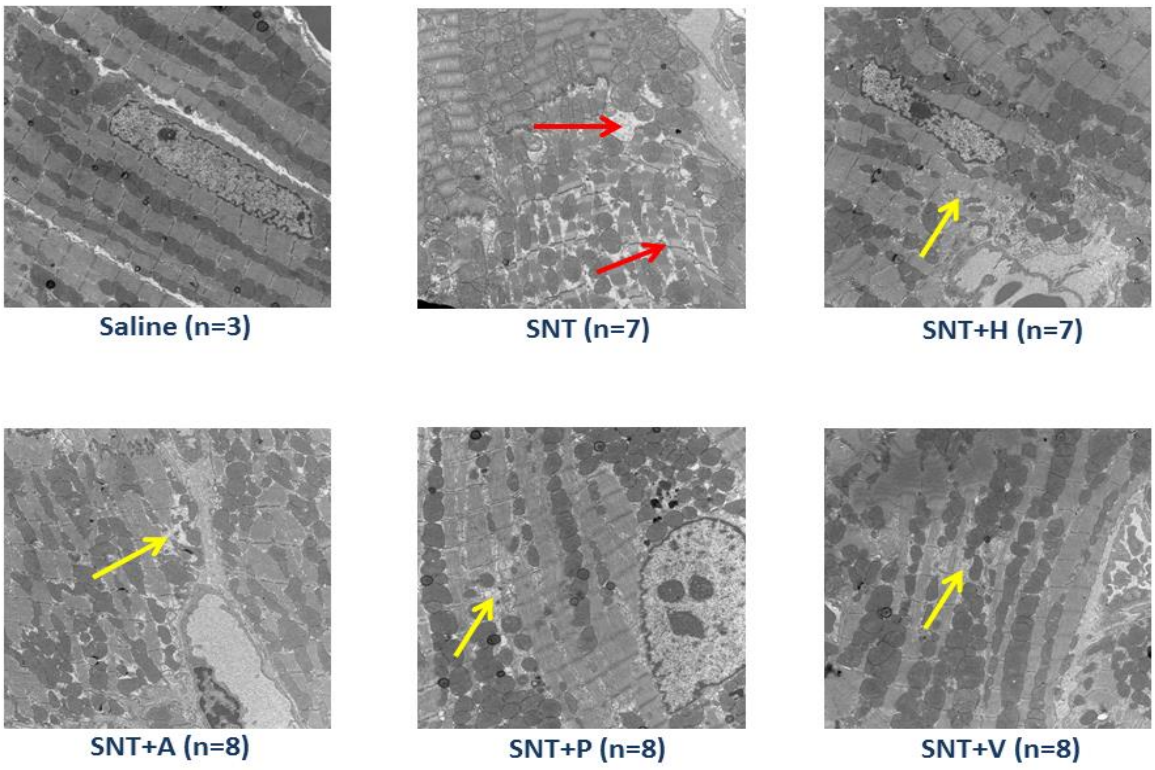

Figure 6: Representative electron microscopy images of heart samples from mice in the SNT treatment arm. Images were taken at 5,800x magnification. Red arrows indicate severe damage and loss of myofibrils. The prophylactic administration of RAS antagonists and H was partially cardioprotective (yellow arrows). SNT, Sunitinib; RAS, renin-angiotensin system; H, Hydralazine; A, Aliskiren; P, Perindopril; V, Valsartan.

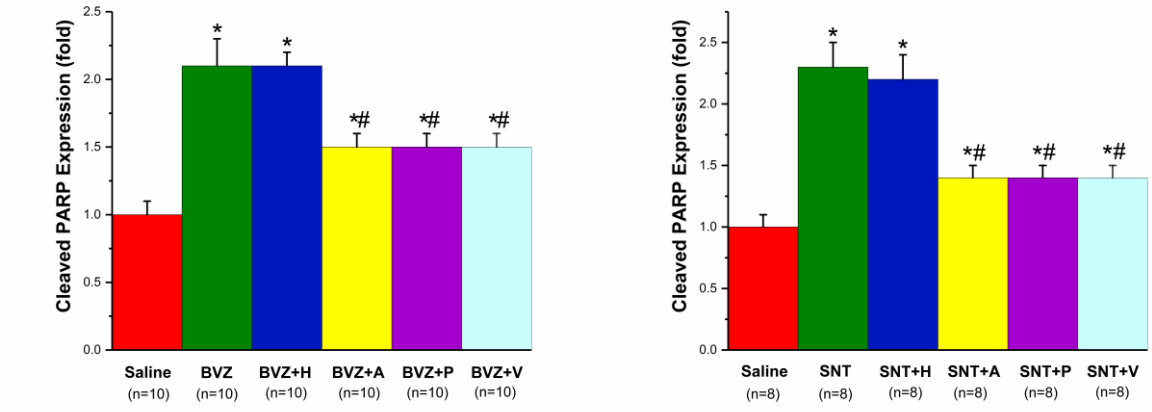

Figure 7: Expression of cleaved PARP protein in C57Bl/6 mice treated with either 0.9% saline, BVZ, SNT, and /or prophylactic administration of Hydralazine or RAS antagonists, on Day 28. The results are reported as mean ± SEM. *p<0.05 as compared to Saline. #p<0.05 as compared to BVZ alone. PARP, Poly (ADP-ribose) polymerase; BVZ, Bevacizumab; RAS, renin-angiotensin system; H, Hydralazine; A, Aliskiren; P, Perindopril; V, Valsartan.