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The Utility of Cardiac Biomarkers and Echocardiography for the Early Detection of Bevacizumab and Sunitinib Mediated Cardiotoxicity

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Abstract

Rationale: The recent introduction of novel anti-cancer therapies, including Bevacizumab (BVZ) and Sunitinib (SNT), is associated with an increased risk of cardiotoxicity. However, early identification of left ventricular (LV) systolic dysfunction may facilitate dose modification and avoid the development of advanced heart failure.

Objective: Using a murine model of BVZ and SNT mediated cardiotoxicity, to investigate whether cardiac biomarkers and/or tissue velocity imaging (TVI) using echocardiography can detect early changes in cardiac function before a decrease in left ventricular ejection fraction (LVEF) is identified.

Methods and Results: A total of 75 wild-type C57Bl/6 male mice were treated with either 0.9% saline, BVZ, or SNT. Serial monitoring of blood pressure, high sensitivity troponin I (hsTnI), and echocardiographic indices were performed over a 14-day study period, after which the mice were euthanized for histological and biochemical analyses. Mice treated with either BVZ or SNT developed systemic hypertension as early as day 7 which increased by day 14. Cardiac biomarkers, specifically hsTnI, were not predictive of early LV systolic dysfunction. Although conventional LVEF values decreased at day 13 in mice treated with either BVZ or SNT, TVI confirmed early LV systolic dysfunction at day 8. Histological and biochemical analysis demonstrated loss of cellular integrity, increased oxidative stress, and increased cardiac apoptosis in mice treated with BVZ or SNT therapy at day 14.

Conclusions: In a murine model of BVZ or SNT mediated cardiomyopathy, non-invasive assessment by TVI detected early LV systolic dysfunction prior to alterations in conventional echocardiographic indices.

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New and Noteworthy

The current basic science study evaluates the utility of cardiac biomarkers and echocardiography using tissue velocity imaging and strain rate imaging for the early detection of Bevacizumab and Sunitinib mediated cardiotoxicity.
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Abbreviation List

100 Blood pressure (BP)
101 Bevacizumab (BVZ)
102 Brain natriuretic peptide (BNP)
103 Colorectal cancer (CRC)
104 C-reactive protein (CRP)
105 Doxorubicin (DOX)
106 Endocardial velocity (Vendo)
107 Fractional shortening (FS)
108 Heart rate (HR)
109 High sensitivity troponin I (hsTnI)
110 Intraperitoneal (i.p.)
111 Intravenous (i.v.)
112 Left ventricular (LV)
113 Left ventricular ejection fraction (LVEF)
114 Left ventricular end-diastolic diameter (LVEDD)
115 Left ventricular end-systolic diameter (LVESD)
116 Mean arterial blood pressure (MAP)
117 Motion mode (M-mode)
118 Nitric oxide (NO)
119 N-terminal prohormone brain natriuretic peptide (NT-ProBNP)
120 Oxidative stress (OS)
121 Oxidized phosphatidylcholine (OxPC)
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123  Posterior wall thickness (PWT)
124  Renal cell cancer (RCC)
125  Standard error of the mean (SEM)
126  Strain rate imaging (SRI)
127  Sunitinib (SNT)
128  Tissue velocity imaging (TVI)
129  Transthoracic echocardiography (TTE)
130  Trastuzumab (TRZ)
131  Troponin-I (TnI)
132  Troponin-T (TnT)
133  Vascular endothelial growth factor (VEGF)
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**Introduction**

Cardio-Oncology is a novel discipline that focuses on the prevention, diagnosis, and management of cancer patients who are at risk of developing cardiovascular complications as a result of their anti-cancer treatment. Despite the beneficial effects of chemotherapy agents in increasing overall survival of cancer patients, cardiotoxicity remains a serious complication of many systemic anti-cancer therapies. (4, 5, 26, 32, 36, 47, 49, 58) The treatment strategy for each patient is individualized, involving a combination of surgical intervention, radiation, chemotherapy, and novel targeted biological therapy.

Two types of targeted therapies widely used for treating metastatic colorectal (CRC) and renal cell cancer (RCC), respectively, are the monoclonal antibody Bevacizumab (BVZ; Avastin®) and the tyrosine kinase inhibitor Sunitinib (SNT; Sutent®). (18, 50) In both normal and neoplastic tissues, including CRC, vascular endothelial growth factor (VEGF) plays an important role in angiogenesis, chemotaxis, and regulation of vascular tone. (19, 33) By binding specifically to VEGF-A and inhibiting its interaction with the VEGF receptor (VEGFR), BVZ prevents the proliferation of endothelial cells and formation of new blood vessel cells, thus inhibiting tumor growth. (18) In combination with non-anthracycline based chemotherapy, BVZ is approved for the treatment of metastatic CRC by decreasing tumor progression and improving overall survival. (22, 29) Additionally, solid tumors including RCC are dependent on the development and expansion of a vascular network to support their growth. As opposed to BVZ which inhibits only VEGF-A and its interaction with VEGFR, SNT is an oral tyrosine kinase inhibitor approved for the treatment of metastatic RCC, that blocks the activity of multiple receptors, including VEGFR 1-3, platelet-derived growth factor receptors (PDGFR) α and β, and AMPK. (10, 21, 26) Despite the beneficial effects of both BVZ and SNT in improving overall
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survival in the CRC and RCC populations, an unexpected side effect of both anti-cancer drugs is
the risk of developing cardiotoxicity in nearly 1 in 4 individuals. (6, 10, 24, 47, 50, 58)

In clinical practice, serial monitoring of cardiac biomarkers and left ventricular ejection
fraction (LVEF) using non-invasive cardiac imaging are important diagnostic tools in the
detection of cardiac dysfunction amongst cancer patients. (3, 7, 8, 14, 15, 35, 52) In the last
decade, the use of cardiac biomarkers, including Troponin-I (TnI), C-reactive protein (CRP), and
brain natriuretic peptide (BNP) have emerged as a more sensitive and specific tool for the early
identification, assessment, and monitoring of cardiotoxicity due to anti-cancer drugs. (1, 7, 8, 34,
35, 42, 52) Recent studies have demonstrated that frequent sampling of TnI and CRP was able to
identify a subset of women with breast cancer at high risk of Doxorubicin and Trastuzumab
(DOX+TRZ) mediated cardiac dysfunction prior to a decrease in LVEF. (7, 44) Whether these
cardiac biomarkers, including high sensitivity troponins, (37) can similarly detect early evidence
of BVZ and SNT mediated cardiac dysfunction, requires further study.

Non-invasive assessment of LVEF using multiple-gated acquisition scintigraphy and
transthoracic echocardiography (TTE) continue to be the most common methods for monitoring
cardiac dysfunction in the cancer setting. (3, 13-16, 44, 51, 54) Despite the established use of
LVEF as a measure of cardiac function, compensatory myocardial reserve enables adequate
ventricular output in the presence of dysfunctional cardiomyocytes, such that the extent of
cardiac injury is often not recognized and/or underestimated at an early time point in treatment.
(14) Therefore, novel echocardiographic parameters including tissue velocity imaging (TVI) and
strain rate imaging (SRI) have been developed to improve the diagnostic value of non-invasive
echocardiography. (13, 14, 16, 28, 44, 51, 59) A number of basic science and clinical studies
have confirmed the role of TVI and SRI for the early detection of DOX+TRZ mediated cardiac
dysfunction in the breast cancer setting. (13, 14, 16, 30, 44, 51) However, little is known about
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whether these novel echocardiographic parameters can be applied in the early detection of BVZ or SNT mediated cardiotoxicity.

The objective of this study is to evaluate whether cardiac biomarkers and/or novel echocardiographic techniques can detect early manifestations of cardiac dysfunction before a reduction in LVEF is identified, in a murine model of BVZ and SNT induced cardiomyopathy.
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**Methods**

1. **Experimental 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 75 C57Bl/6 male mice (8-10 weeks old; Jackson Laboratories, ME, US) were randomized into 1 of 3 treatment groups including: (i) 0.9% saline [intraperitoneal (i.p.) n=5]; (ii) BVZ [10 mg/kg, intravenously (i.v.) n=35]; or (iii) SNT [40 mg/kg/day, orally, n=35]. (9, 11, 21, 23) Each animal underwent baseline TTE prior to administration of the targeted biological agent. A single i.p. injection of saline or i.v. injection of BVZ (10 mg/kg: Hoffman-La Roche Ltd.) was administered following baseline data acquisition. Bevacizumab is a recombinant monoclonal antibody which targets VEGF-A with one additional murine complementary determining region, which provides cross-reactivity with murine receptors. SNT (40 mg/kg/d: Pfizer Canada Ltd.) dissolved in saline was administered via daily oral gavage for a total of 14 days. As validated by Chu et al. and Chen et al., the BVZ and SNT dosages used in our murine model produce blood concentrations comparable to those observed in the clinical settings of CRC and RCC. (9, 11) Serial TTE was performed daily for 14 days, at which time all surviving mice were euthanized (150 mg/kg pentobarbital buffered with 2% lidocaine i.p.) and the hearts were preserved for histological and biochemical analyses.
2. Murine echocardiography

Echocardiographic data was collected using a 13-MHz probe (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) with TVI capability. All 75 C57Bl/6 mice were awake and underwent TTE at baseline and were followed daily thereafter for 14 days, as previously described. (2, 30, 40, 41, 55, 56, 60) Each mouse was imaged in the parasternal long axis and short axis windows, in addition to acquisition of 3 different frames of M-mode echocardiography for the non-invasive determination of LV morphology and function. (2, 30, 40, 41, 55, 56, 60)

LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and posterior wall thickness (PWT) were measured. Left ventricular ejection fraction (LVEF) was calculated by measurement of LV end-systolic and end-diastolic volumes using the prolate ellipsoid geometric model. (57) Endocardial velocity ($V_{endo}$) was calculated using TVI, which was acquired in the short axis window at the level of the papillary muscles, at a rate of 483 frames/s. (30, 46, 55, 60)

For peak $V_{endo}$, a region of interest (0.2 x 0.2 mm) was manually positioned along the posterior wall of the endocardium. Radial SR was measured over an axial distance of 1 mm (width, 0.6 mm) and the temporal smoothing filters were turned off for all measurements. (30, 46, 55, 60)

Post-processing of all images was conducted offline using the EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US). The echocardiographic observers were blinded to the various treatment groups.

To assess the variability of $V_{endo}$ and radial SR, a total of 30 mice were randomly chosen from the various treatment groups. Both $V_{endo}$ and radial SR measurements were performed independently by a single observer (D.S.J.), two weeks apart, to determine intra-observer variability. Inter-observer variability was determined from echocardiographic measurements that were processed separately by two independent observers (K.A.B. and D.S.J.).
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3. Hemodynamics

Non-invasive measurements of heart rate (HR) and blood pressure (BP) were performed in all 75 conscious, restrained mice via a tail cuff method (CODA system, High Throughput, Kent Scientific, Torrington, CT), as previously described. (2, 17) 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, day 7, and day 14, BP measurements were collected, from which average values for mean arterial pressure (MAP) were calculated.

4. Cardiac biomarkers: hsTnI

Blood was collected via the internal jugular vein in all 75 animals at baseline, day 7, and day 10. Upon euthanasia at day 14, the heart was removed and all blood remaining in the thoracic cavity was immediately collected through pipette. Serum high sensitivity troponin I (hsTnI) was quantified using a mouse-specific enzyme-linked immunosorbent assays (Life Diagnostics, Inc. Cat. No. 2010-1-HS) and the absorbance was read at 450 nm using a microplate reader (MRX Microplate Reader, Dynex Technologies Inc. 1CXD-4588, Chantilly, VA, US).

5. Histological Analysis

A total of 35 mice (n=5 controls; n=15 for BVZ; and n=15 for SNT) were euthanized at day 8 and day 14 for electron microscopy (EM) studies. After the chest cavity was rapidly opened and the major blood vessels and connective tissue were removed, the heart was blotted dry, weighed, and the heart weight/body weight ratio was calculated. Half of the LV was sectioned for histological analysis via EM, as previously described. (30, 55, 60) Samples were dehydrated in ascending concentrations of ethanol and embedded in Epon 812 using standard techniques. Thin sections were stained with uranyl acetate and lead citrate, viewed, and
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photographed on a Philips CM12 electron microscope to determine the extent of cell degradation.

6. Oxolipidomic analysis

A total of 35 mice (n=5 controls; n=15 for BVZ; and n=15 for SNT) were euthanized at day 8 and day 14 for oxidized phosphatidylcholine (OxPC) studies. OxPC, a marker of inflammation and oxidative stress (OS), was quantified using liquid chromatography and electrospray ionization tandem mass spectrometry. Briefly, lipid was extracted from LV myocardial tissue in congruence with the protocol adapted from Folch et al. in the presence of 9:0-9:0 phosphatidylcholine as an internal standard. Oxolipidomics analysis was carried on a reverse phase high-performance liquid chromatography using an Ascentis Express C18 column (15 cm x 2.1 mm, 2.7 μm; Supelco Analytical, Bellefonte, PA). Elution was performed using a linear gradient of solvent A (acetonitrile-water, 60:40 vol/vol) and solvent B (isopropanol-acetonitrile, 90:10, vol/vol). Both of the solvents contained 10 mM ammonium formate and 0.1% formic acid with a flow rate of mobile phase at 0.260 mL/min. The time program used was 0.01 minute 32% B; 1.50 minutes 32% B; 4.00 minutes 45% B; 5.00 minutes 52% B; 8.00 minutes 58% B; 11.00 minutes 66% B; 14.00 minutes 70% B; 18.00 minutes 75% B; 21.00 minutes 97% B; 25.00 minutes 97% B; 25.10 minutes 32% B, and 30.00 minutes 32% B. The elution was stopped at 30.10 minutes. Auto-oxidized 16:0-18:2 phosphatidylcholine and 16:0-20:4 phosphatidylcholine were used to generate an assigned theoretical structure corresponding to the molecular weight. Oxidation products were separated from nonoxidized phosphatidylcholine allowing for correct determination of OxPC molecules in samples. Data were collected using Analyst 1.6 software (Applied Biosystems, Ontario, Canada) and quantified using MultiQuant 2.1 (AbSciex, Ontario, Canada).
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7. Protein analysis: Apoptotic markers

A total of 65 mice (n=5 controls; n=20 for Doxorubicin (DOX); n=20 for BVZ; and n=20 for SNT) were used for Western analysis of apoptotic markers at day 14. Mice treated with a single i.p. injection of DOX (20 mg/kg) were used as a positive control for increased cardiac apoptosis, as previously described (30, 60). Frozen heart tissue was ground in liquid nitrogen and proteins were extracted in the radioimmunoprecipitation buffer containing protease and phosphatase inhibitors (Thermo Scientific). A total of 30 μg of protein was loaded and electrophoresed in 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Roche Diagnostics). (30, 38, 39, 55, 60) Membranes were incubated with the primary antibody to Caspase-3, Bax, and PARP (Cell Signaling) overnight at 4°C. Anti-rabbit (Cell Signaling) secondary antibody was used to detect the primary antibody and was accomplished using the ECL Plus detection reagent (Western Lightning Plus-ECL, Amersham). For the loading control, a polyclonal antibody to GAPDH (Sigma) was used. Band intensities were quantified using image analysis software (Quantity One; BioRad Laboratories, Inc). (30, 38, 39, 55, 60)

8. 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
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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.
Results

1. Conventional echocardiographic, TVI, and SR imaging parameters

Heart rate, LV cavity dimensions, and systolic function were similar at baseline between all treatment groups. In mice receiving either BVZ or SNT, there were no overt visible behavioral changes nor weight loss during the 14 day study. Heart weight/body weight ratio, heart rate, and PWT remained within normal limits throughout the duration of the 14-day study for all treatment groups (Table 1). In both BVZ and SNT treated mice, conventional echocardiographic indices showed a significant increase in LVEDD and a decrease in LVEF beginning at day 13 (Figures 1-2). 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. Similarly, in the SNT treatment group, LVEDD increased from 3.1±0.2mm at baseline to 3.9±0.3mm at day 14 (Figure 1, Table 1). Both BVZ and SNT treatment groups demonstrated a decrease in conventional LVEF by day 13 of the study (Figure 2).

Both BVZ and SNT treatment groups demonstrated normal \( V_{endo} \) and SR values at baseline. TVI parameters, including \( V_{endo} \) and SR, decreased at day 8 in both BVZ and SNT treatment groups (Figures 3-4). In mice treated with BVZ, \( V_{endo} \) decreased from 3.5±0.3cm/s at baseline to 2.4±0.1cm/s as early as day 8, and continued to decrease to 1.3±0.1cm/s at day 14. Radial SR 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 3-4). 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, radial 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 (Figures 3-4).
The intra- and inter-observer agreement rates for $V_{\text{endo}}$ were 0.1±0.05 cm/s and 0.2±0.05 cm/s, respectively. The intra- and inter-observer agreement rates for SR were 0.8±0.4 s$^{-1}$ and 0.9±0.3 s$^{-1}$, respectively.

2. Hemodynamics

Baseline measurements of MAP were within normal limits for all treatment groups. At days 7 and day 14, the MAP of saline treated mice remained unchanged, as compared to baseline. Mice treated with BVZ demonstrated a significant increase in MAP from 90±2 mmHg at baseline, to 120±5 at day 7, to 141±5 mmHg at day 14 (p<0.05) (Figure 5). Similarly, mice treated with SNT demonstrated a significant increase in MAP from 89±3 mmHg at baseline, to 115±6 at day 7, to 135±4 mmHg at day 14 (p<0.05) (Figure 5).

3. Cardiac biomarkers: hsTnI

All mice demonstrated non-detectable hsTnI values at baseline, day 7, and day 10. In animals treated with either BVZ or SNT, the hsTnI 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).

4. Electron microscopy

Approximately 15,000 cells were scanned from 3 randomly derived blocks of myocardial tissue and evaluated for dilation of the sarcoplasmic reticulum and loss of cell integrity. At day 8, there was no evidence of cellular injury in mice treated with BVZ or SNT as compared to controls. However, at day 14, BVZ and SNT treated animals demonstrated an increased loss of cellular integrity and myofibril disarray (Figure 6). There was no statistical difference, however, between the BVZ and SNT treatment groups at day 14.

5. Oxidative stress and apoptotic markers
A total of 82 distinct OxPC studies were evaluated in each experimental group at days 8 and 14. There was no discernable change in OxPC in mice treated with either BVZ or SNT at day 8 as compared to controls. At day 14, however, the heat map generated for the most abundant OxPC molecular demonstrated a 10-fold increase in OxPC molecules in mice treated with either BVZ or SNT as compared to controls (Figures 7A and B). Western blot analysis demonstrated evidence of apoptotic cell death and Caspase-3 cleavage amongst BVZ and SNT treated animals at day 14 (p<0.05) (Figure 8). Mice treated with either BVZ or SNT demonstrated 2.5-fold and 2-fold increases in Caspase-3 levels, respectively (p<0.05). As compared to saline treated animals, there was no significant evidence of an increase in either Bax or PARP expression at day 14 in mice treated with BVZ or SNT (data not shown).
Discussion

An increased understanding of the biology of cancer has allowed for the development of new effective therapies that utilize various pathways to suppress tumor growth, including the use of the targeted agents BVZ and SNT. Although effective against CRC and RCC in reducing overall morbidity and mortality, these novel anti-cancer drugs are associated with an increased risk of developing cardiotoxicity. (6, 11, 18, 22, 24, 25, 43, 45, 50, 53, 61) The aim of the current study was to determine the utility of cardiac biomarkers and TVI parameters for the early detection of BVZ and SNT mediated cardiotoxicity, potentially avoiding the development of advanced heart failure. In a murine model of BVZ and SNT mediated cardiac dysfunction, we demonstrated: i) development of systemic hypertension; ii) serial hsTnI was unable to detect early myocardial dysfunction; iii) TVI and SRI parameters were able to detect LV systolic dysfunction 5 days earlier than traditional LVEF parameters; and iv) evidence of loss of cellular integrity with increased OS and apoptosis.

BVZ and SNT mediated hypertension

The use of novel anti-angiogenic drugs including BVZ and SNT result in the development of systemic hypertension as outlined in several basic science and clinical studies. (10, 12, 18, 31, 43, 45, 50, 53) Both agents inhibit the VEGF pathway, which causes down-regulation of endothelial nitric oxide (NO) synthase expression and decreased synthesis of endothelial NO. This results in systemic vasculature constriction and development of hypertension. (27, 31, 48) In an acute murine model using Swiss-Webster male mice, Chu et al. observed no change in blood pressure over a 12-day period among animals administered SNT. (11) Conversely, Kappers et al. observed an increase in MAP by day 6 in male Wistar-Kyoto rats treated with SNT, (31) and Curwen et al. observed an increased in diastolic blood pressure at day
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458 4 in male Wistar rats treated with Cediranib, a VEGF inhibitor. (12) Similarly, in our current study, C57Bl/6 male mice treated with either BVZ or SNT developed an increase in MAP as early as day 7, which continued to increase at day 14 by nearly 50% as compared to baseline.

459 Although increased LV wall thickness is often associated with increased afterload, our study demonstrated no change in LV wall dimensions throughout the study. 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. (2) Additionally, as there was no overall change in heart weight/body weight ratio in mice treated with either BVZ or SNT, it is plausible that these two anti-cancer drugs may interfere with the adaptive cardiac hypertrophy that occurs in the setting of increased afterload. Therefore, future animal studies are warranted to elucidate the subsequent hemodynamic side effects of chronic administration of BVZ and SNT in a murine model.

Cardiac Biomarkers: Troponin I

Cardiac troponins including TnI and TnT, are cardiac regulatory proteins, which are involved in the calcium-mediated interaction of actin and myosin. Troponins are the gold standard for the measurement of cardiac dysfunction due to their sensitivity and specificity for detecting subtle myocardial necrosis. (37) Although previous clinical studies have validated the use of serial troponins in the early detection of cardiotoxicity following DOX+TRZ treatment in the breast cancer setting. (7, 44) little is known about the use of these cardiac biomarkers in the setting of BVZ and SNT mediated cardiac dysfunction. In a murine model of BVZ mediated cardiotoxicity, Chen et al. demonstrated an increase in TnI serum levels by nearly 2 fold in mice following 3 weeks of treatment with BVZ. (9) Our study adds to the paucity of existing literature that exists on the potential predictive value of high sensitivity troponins for the early detection of cardiac dysfunction in a murine model of BVZ and SNT mediated cardiotoxicity. Although we have confirmed evidence of myocardial necrosis in mice receiving either BVZ or SNT at day 14,
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the ability of hsTnI to serve as an early predictive marker of cardiotoxicity was not validated, as
the cardiac biomarker was undetectable at days 7 and 10. Further investigations are warranted to
evaluate the role of cardiac biomarkers for the early detection of BVZ and SNT mediated
cardiotoxicity in the clinical setting.

Early detection of cardiotoxicity using TVI

Traditional monitoring of cancer patients receiving anti-cancer therapy involves the
measurement of LVEF through the use of serial MUGA scans or TTE throughout the treatment
cycle. (3, 13-16, 44, 51, 54) In addition to the high degree of intra- and inter-observer variability
that often accompanies conventional echocardiographic analyses, (13) LVEF measurement is a
relatively insensitive parameter for the early detection of drug-mediated cardiotoxicity. (4) Once
LVEF falls below 40% in a cancer patient, irreversible cardiac injury may have already occurred,
thereby precluding any chance of prevention. (4) The novel use of TVI has recently been
established to supplement conventional echocardiography in the evaluation of myocardial
dysfunction in the Cardio-Oncology setting. (13, 14-16, 28, 44, 51, 59) TVI-derived parameters
are proven to be less influenced by hemodynamic variables, and provide more precise and
reproducible analysis of both systolic and diastolic function. (13, 14-16, 28, 44, 51, 59) In an
animal-based model of DOX mediated cardiac dysfunction, Neilan et al. demonstrated that early
changes in TVI-derived parameters were predictive of the late development of cardiac
dysfunction and increased mortality. (4) In an acute murine model of DOX+TRZ mediated
cardiac dysfunction, we previously demonstrated that although LVEF decreased at day 5, TVI
was significantly decreased in both V_{endo} and radial SR as early as 24 hours following treatment.
(30) However, the role of TVI for the early detection of BVZ and SNT induced cardiotoxicity in
an animal model has not been previously explored. In the current study, mice treated with BVZ
or SNT demonstrated an increase in LV cavity dimensions and a decrease in conventional echo
parameters, including LVEF, at day 13. In contrast, \( V_{\text{end}} \) and radial SR decreased 5 days earlier in mice receiving either BVZ or SNT, confirming that TVI is a sensitive and reproducible measure of early cardiac dysfunction. We hypothesize as BVZ and SNT both interrupt the VEGF pathway, \((9, 11, 19, 21, 23, 43, 61)\) this common feature may explain why there was a similar decline in cardiac function at the same time point. 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. However, these results require validation in the clinical setting of CRC and RCC patients treated with BVZ and SNT, respectively.

**Mechanisms of BVZ and SNT mediated cardiotoxicity**

The precise underlying mechanisms of BVZ and SNT mediated cardiotoxicity have yet to be fully elucidated. In an animal model of age-matched, wild-type Swiss-Webster mice treated with 40 mg/kg/d of SNT, Chu et al. demonstrated mitochondrial swelling and degenerative changes in cardiomyocytes using transmission electron microscopy at day 12. \((11)\) Our EM findings corroborate this previous study, as changes in cellular integrity do not occur early at day 8, but rather later at day 14. Additionally, in a chronic 6 month model involving mice treated with BVZ 10 mg/kg i.v. every 2 weeks, there was evidence of increased cardiac fibrosis as measured by hydroxyproline content, in comparison to control EndoCD/5-FC treatment groups. \((9)\) Our study validates the significant loss and disruption of cardiac myofibrils in BVZ and SNT treated animals. These findings may be indicative of cellular damage caused by impaired ATP generation due to mitochondrial dysfunction. An important finding from our current study is the absence of cardiac fibrosis, suggesting that the dysfunction observed may be reversible.

Although BVZ inhibits VEGF-A alone and SNT inhibits multiple receptors including VEGFR 1-3, PDGFR \( \alpha \) and \( \beta \), and AMPK, \((10, 18, 21, 26)\) it is plausible that the cardiotoxic side effects of these two different anti-cancer agents may result in the activation of common
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downstream apoptotic and OS pathways. Although a few studies have previously evaluated the relationship between SNT mediated cardiotoxicity and increased apoptosis, (11, 27) there is a paucity of data on BVZ induced cardiac dysfunction and this pathway. Apoptosis occurs through inhibition of AMPK signaling (primarily affected by SNT), 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). (30, 38, 39, 55, 60) In an animal model of SNT induced cardiomyopathy, Chu et al. demonstrated the direct targeting of myocardial mitochondria, resulting in cytochrome-C release and Caspase-9 activation. (11) Corroborating these findings, although there was no increase in Bax and PARP expression in our study, there was a significant increase in Caspase-3 protein and OxPC levels among BVZ and SNT treated animals. It is plausible to propose that the apoptotic events induced by BVZ and SNT are not solely mediated through the Bax/Bcl-xL pathway. In support of this hypothesis, Hasinoff et al. demonstrated that in ventricular myocytes treated with SNT, Bax levels were not significantly changed, indicative of the inactivity of this pathway in the induction of TKI-induced apoptosis. (27) However, this group also demonstrated that levels of caspase-3 and caspase-7 levels rapidly increased following SNT treatment, indicative of the major contribution of caspase in the development of cardiotoxicity. (27) In the future, additional basic science and clinical trials are needed to thoroughly elucidate the underlying mechanisms involved in the myocardial dysregulation, OS, and apoptosis observed in this unique population of BVZ and SNT mediated heart failure.

Limitations

There are a few limitations to our study. The current study characterized drug-induced cardiac dysfunction in an acute murine model of BVZ and SNT mediated heart failure. As these drugs are administered over a period of several months in patients with metastatic CRC or RCC,
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it would be useful to design a chronic murine model of BVZ and SNT mediated cardiac
dysfunction, to more closely mimic the clinical setting. Second, we exclusively evaluated
changes in cardiac hsTnI. In future studies, the evaluation of various cardiac biomarkers,
including cardiac hsTnT and NT pro-BNP should be investigated for their predictive role of
detecting early cardiac dysfunction. Finally, there may be significant gender-based differences in
the cardiotoxic side effects of BVZ and SNT. As the current study focused on C57Bl/6 male
mice, future studies evaluating BVZ and SNT- mediated cardiotoxicity in female mice are
warranted.
Conclusion

This novel study demonstrated that TVI can detect early LV systolic dysfunction prior to alterations in conventional echocardiographic parameters in an acute murine model of BVZ and SNT induced cardiomyopathy. Future clinical studies are required to investigate the potential use of cardiac biomarkers and TVI parameters for the early detection of subclinical alterations in cardiac function among CRC and RCC patients treated with either BVZ or SNT, potentially avoiding the development of advanced heart failure.
Acknowledgments

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Figure Legends

Figure 1: Echocardiography: Left ventricular end diastolic diameter [LVEDD (mm)] values in mice treated with saline, BVZ, and SNT. LVEDD increased at day 13 in BVZ and SNT-treated mice. All data are expressed as mean±SD. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib.

Figure 2: Echocardiography: Left ventricular ejection fraction [LVEF (%)] of saline, BVZ-, and SNT-treated mice as determined by M-mode echocardiography. LVEF decreased at day 13 in BVZ and SNT-treated mice. All data are expressed as mean±SD. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib.

Figure 3: Echocardiography: Peak endocardial systolic velocity [V_endo (cm/s)] in mice treated with saline, BVZ, and SNT. V_endo decreased in BVZ and SNT mice 8 days post-treatment. All data are expressed as mean±SD. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; V_endo, peak endocardial systolic velocity.

Figure 4: Echocardiography: Radial strain rate [SR (s⁻¹)] values in mice treated with saline, BVZ, and SNT. SR decreases in BVZ and SNT mice after 8 days. All data are expressed as mean±SD. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; SR, radial strain rate.

Figure 5: Hemodynamics: Mean arterial blood pressures at baseline, day 7, and day 14 in mice treated with BVZ and SNT, as compared to saline controls. BVZ, Bevacizumab; SNT, Sunitinib. All data are expressed as mean±SD. *p<0.05 between baseline and day 7 for BVZ; **p<0.05 between both baseline and day 7 as compared to day 14 for BVZ; #p<0.05 between baseline and day 7 for SNT; ##p<0.05 between both baseline and day 7 as compared to day 14 for SNT.
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**Figure 6: Histology:** Electron microscopy of representative samples from mice treated with saline, BVZ, or SNT at day 14 (5800X magnification). Arrows indicate disruption and loss of myofibrils of cardiomyocytes in the BVZ and SNT treatment groups, as compared to controls. BVZ, Bevacizumab; SNT, Sunitinib.

**Figure 7: Oxidized Phospholipids:** (A) Heat map graphic demonstrating changes of each OxPC species in animals treated with saline (n=5), BVZ (n=10), or SNT (n=10) at day 14. (B) Total mass of OxPC/mg of heart tissue extracted from male mice treated with either BVZ or SNT, as compared to controls at day 14. BVZ, Bevacizumab; OxPC, oxidized phosphatidylcholine; SNT, Sunitinib. All data are expressed as mean±SD. *p<0.05 comparing BVZ to control; **p<0.05 comparing SNT to control.

**Figure 8: Apoptosis:** Western blot gel (A) and data (B) demonstrating positive expression change of Caspase-3 in mice treated with saline, DOX (positive control), BVZ, and SNT. (Data for Bax and PARP proteins not shown). BVZ, Bevacizumab; DOX, Doxorubicin; SNT, Sunitinib. All data are expressed as mean±SD. *p<0.05 comparing DOX to control; **p<0.05 comparing BVZ to control; #p<0.05 comparing SNT to control.
Tables

<table>
<thead>
<tr>
<th>Morphological and echocardiographic variables</th>
<th>Group</th>
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<th>Day 14</th>
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Table 1: Morphological and echocardiographic data at baseline and day 14 (mean ± SD) in mice receiving either 0.9% saline, BVZ, or SNT treatment. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; HR, heart rate; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter.
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ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; \( V_{endo} \), peak endocardial systolic velocity; SR, strain rate.
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Table 2: Cardiac biomarker hsTnI baseline values for all treatment groups were below detectable limits. Day 14 values are recorded as mean ± SD in ng/mL. *p<0.05 as compared to baseline. BVZ, Bevacizumab; SNT, Sunitinib; hsTnI, high sensitivity Troponin I.
A

<table>
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<tr>
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<tr>
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<td>GAPDH 36 kD</td>
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B

![Bar chart showing Caspase 3 levels across different treatments: Saline, DOX, BVZ, and SNT.](image)