Sorafenib Cardiotoxicity Increases Mortality After Myocardial Infarction

Jason M. Duran,* Catherine A. Makarewich,* Danielle Trappanese, Polina Gross, Sharmeen Husain, Jonathan Dunn, Hind Lal, Thomas E. Sharp, Timothy Starosta, Ronald J. Vagnozzi, Remus M. Berretta, Mary Barbe, Daohai Yu, Erhe Gao, Hajime Kubo, Thomas Force, Steven R. Houser

Rationale: Sorafenib is an effective treatment for renal cell carcinoma, but recent clinical reports have documented its cardiotoxicity through an unknown mechanism.

Objective: Determining the mechanism of sorafenib-mediated cardiotoxicity.

Methods and Results: Mice treated with sorafenib or vehicle for 3 weeks underwent induced myocardial infarction (MI) after 1 week of treatment. Sorafenib markedly decreased 2-week survival relative to vehicle-treated controls, but echocardiography at 1 and 2 weeks post MI detected no differences in cardiac function. Sorafenib-treated hearts had significantly smaller diastolic and systolic volumes and reduced heart weights. High doses of sorafenib induced necrotic death of isolated myocytes in vitro, but lower doses did not induce myocyte death or affect inotropy. Histological analysis documented increased myocyte cross-sectional area despite smaller heart sizes after sorafenib treatment, further suggesting myocyte loss. Sorafenib caused apoptotic cell death of cardiac- and bone-derived c-kit+ stem cells in vitro and decreased the number of BrdU+ (5-bromo-2'-deoxyuridine+) myocytes detected at the infarct border zone in fixed tissues. Sorafenib had no effect on infarct size, fibrosis, or post-MI neovascularization. When sorafenib-treated animals received metoprolol treatment post MI, the sorafenib-induced increase in post-MI mortality was eliminated, cardiac function was improved, and myocyte loss was ameliorated.

Conclusions: Sorafenib cardiotoxicity results from myocyte necrosis rather than from any direct effect on myocyte function. Surviving myocytes undergo pathological hypertrophy. Inhibition of c-kit+ stem cell proliferation by inducing apoptosis exacerbates damage by decreasing endogenous cardiac repair. In the setting of MI, which also causes large-scale cell loss, sorafenib cardiotoxicity dramatically increases mortality. (Circ Res. 2014;114:1700-1712.)

Key Words: cell death ■ metoprolol ■ myocardial infarction ■ sorafenib ■ stem cells

Protein kinase inhibitors predominantly targeting mutated tyrosine kinases (but also serine/threonine kinases) have revolutionized cancer therapy during the past decade. Several malignancies that were formerly fatal are now more manageable chronic diseases thanks to these agents. However, several kinase inhibitors have been associated with significant cardiovascular toxicities, including contractile dysfunction and heart failure, as well as vascular events.2,3 Added to this, patients receiving kinase inhibitors are living to older ages, further increasing risk of cardiovascular complications. This has led to the creation and expansion of the field of cardio-oncology.2

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The most problematic agents to date are the so-called vascular endothelial growth factor (VEGF) signaling pathway inhibitors. These agents are associated with hypertension that can be severe in some patients.4 The approved agents include axitinib, pazopanib, regorafenib, sunitinib, sorafenib, and vandetanib, and others are under development. These agents tend to be poorly selective, inhibiting several kinases that play no role in malignancies. Sunitinib was the first of this group shown to cause left ventricle dysfunction and heart failure in patients.5,6 However, molecular mechanisms were difficult to identify fully because of the poor selectivity of this drug. Another popular agent is sorafenib (Nexavar, Bayer; Leverkusen, Germany), so named because it targets (among other kinases) the serine/threonine kinase RAF and the related B-RAF.7 These kinases are implicated in several malignancies including renal cell carcinoma, hepatocellular carcinoma (HCC), and melanoma. Several groups have been unable to identify the mechanisms of sorafenib...
cardiotoxicity. The goal of the present study was to determine the bases of this cardiotoxicity and potentially remedy it, not just for sorafenib but for other problematic agents as well.

Sorafenib gained Food and Drug Administration approval for treatment of RCC in 2005. Sorafenib has known antagonism against B-RAF and RAF1 (early kinases in the MAP-kinase cascade) as well as against VEGF receptor and platelet-derived growth factor receptor, making it particularly well suited for the treatment of RCC. Sorafenib is also known to inhibit c-kit, a receptor that is upregulated in other cancers such as gastrointestinal stromal tumors. The c-kit receptor is also expressed on other receptors that are known targets of sorafenib.

In the first randomized, controlled, phase III clinical trial of sorafenib for treatment of RCC, a significant increase in cardiotoxicity was reported in the sorafenib treatment arm versus placebo (2.7 versus 0.04%, \( P = 0.01 \)). Interestingly, the occurrence of symptoms related to heart failure was not significantly different between the placebo and treatment arms of this study (2% of patients in each group reported dyspnea). This is consistent with the inadequacy of patient self-reporting as a means to detect cardiotoxicity, and it may explain why earlier phase I and II trials of the drug failed to detect new onset heart disease because few of these studies examined cardiac function and proliferation of the tumor. Sorafenib has been shown to inhibit tumor growth in mouse models. Preclinical trials showed that doses of 30 mg/kg/d in mice effectively reduced the proliferation of VEGF-dependent tumors in mice. This dose at inhibiting cancer growth in mouse models. Preclinical trials have demonstrated that cancer patients undergoing therapy with sorafenib and at risk of cardiovascular complications might benefit from prophylactic therapy with \( \beta \)-adrenergic receptor antagonists.

Methods

Sorafenib and Metoprolol Dosing

For all in vivo experiments, doses of 30 mg/kg/d sorafenib were used based on preclinical mouse models that demonstrated efficacy of this dose at inhibiting cancer growth in mouse models. Preclinical trials showed that doses of 30 mg/kg/d in mice effectively reduced the proliferation of VEGF-dependent tumors in mice. This dose blocked growth and proliferation of both RCC and hepatocellular carcinoma in vivo in mouse models. Doses below 30 mg/kg/d were not effective at inhibiting tumor growth in the same mouse models. In humans, phase II and III clinical trials have demonstrated that 400 mg twice daily (800 mg/d) is the most effective dose for these same malignancies (hepatocellular carcinoma and RCC), and this dose achieves a plasma concentration of 2 to 6 g/L or about 1 to 3 mmol/L. Plasma levels of sorafenib were attained by liquid chromatography/mass spectrometry. These same clinical trials have also reported cardiotoxicity at this same dose. For our in vitro experiments, 0.1 to 50 \( \mu \)mol/L sorafenib was used. These in vitro doses encompass the range of those used in preclinical studies during the development of sorafenib. For in vivo metoprolol dosing, animals received 20 mg/kg/d metoprolol tartrate (Sigma-Aldrich, St. Louis, MO). This is a similar IP dose of metoprolol that has been used in...
previously published mouse models to affect airway reactivity and reduce post-MI left ventricle dysfunction in a mouse MI model.

Please refer to the Materials and Methods section in the Online Data Supplement for additional experimental methods.

**Results**

Sorafenib Treatment Increased Post-MI Mortality and Reduced Heart Size But Did Not Affect Cardiac Function

Mice underwent sham operation with vehicle (n=16) or 30 mg/kg/d IP sorafenib (n=10), or animals underwent induced MI with vehicle (n=25), 30 mg/kg/d (n=49), or 40 mg/kg/d (n=20) IP sorafenib dosing. All animals underwent baseline echocardiography prior to dosing, and then each received vehicle or sorafenib for a total duration of 3 weeks. After the first week of dosing, surgery was performed, and the remaining animals continued to receive vehicle or sorafenib for 2 more weeks or until death or euthanasia (Figure 1A). Both sham groups (with vehicle or sorafenib dosing) demonstrated 100% 2-week survival, whereas the MI+Vehicle control group had 40.0% 2-week survival. A log-rank test of the Kaplan–Meier survival analysis showed that sorafenib treatment at the low and high doses significantly reduced survival to 11.7% (P<0.01) and 7.5% (P<0.01), respectively, relative to vehicle-treated MI controls.

Representative echocardiograms taken at baseline, after 1 week of sorafenib treatment (prior to surgery), and 2 weeks post MI+Vehicle or post MI+Sorafenib are displayed in Figure 1B. Mean cardiac function, volumes, and gross heart weight (HW), and HW/tibia length are displayed in Figure 1C. Sorafenib treatment had little effect on cardiac function. After 1 week of sorafenib treatment (before any surgery was performed), both left ventricular ejection fraction and fractional shortening (FS) were slightly diminished in sorafenib-treated animals relative to vehicle-treated controls. Additionally, the sham-operated group receiving sorafenib dosing continued to have slightly depressed left ventricular ejection fraction and fractional shortening relative to the vehicle-treated sham group at 1 and 2 weeks after surgery. None of these effects reached statistical significance. In addition, at 1 or 2 weeks after surgery, there were no significant alterations in cardiac function measured by transthoracic echocardiography in the MI+Vehicle and MI+Sorafenib groups.

The most significant differences between the sorafenib- and vehicle-treated groups were in the cardiac volumes and gross HWs. Animals treated with sorafenib had significantly smaller...
hearts (Figure 1C). End-diastolic and end-systolic volumes in MI+Sorafenib hearts were significantly smaller at 1 and 2 weeks post MI versus MI+Vehicle controls. After 2 weeks, their volumes were so much smaller that they were not significantly greater than those in the sham-operated groups. Similar patterns were observed when examining gross HW and HW normalized to tibia length. The MI+Vehicle control group had increased HWs by 2 weeks post MI consistent with post-MI cardiac remodeling with pathological hypertrophy. These changes were not observed in the MI+Sorafenib group. In fact, MI+Sorafenib mice were not significantly different from Sham+Vehicle controls. Interestingly, when examining HW and HW/tibia length, Sham+Sorafenib animals had smaller HWs than both the MI+Sorafenib and Sham+Vehicle groups. These data suggest that sorafenib had little or no direct effect on myocyte function. However, sorafenib decreased heart size both in the presence and absence of MI injury.

**Sorafenib Induces Myocyte Necrosis at High Doses and Does Not Affect Inotropy of Isolated Myocytes In Vitro**

Sorafenib had little or no effect on baseline cardiac function in vivo. However, regulatory factors controlling overall cardiac pump function could have masked direct negative inotropic effects of sorafenib on myocyte contractile function. To test for this possibility, adult feline left ventricular myocytes were isolated and exposed to 0.1 to 50 μmol/L sorafenib or dimethyl sulfoxide (DMSO) (control). Myocytes were cultured for 72 hours in the presence of the drug, and their survival and contractile function were measured. Figure 2A shows representative bright-field micrographs taken after 72 hours of exposure to sorafenib, and Figure 2B demonstrates survival of myocytes for the first 36 hours in culture. At low concentrations (0.1–5 μmol/L), sorafenib had no effect on myocyte survival, but at the higher concentrations (10–50 μmol/L), sorafenib induced complete myocyte necrosis within the first 12 hours in vitro. Because of the potent necrotic effect observed above 10 μmol/L, cell physiology could only be studied at the lower doses. There were no significant differences in fractional shortening (Figure 2C and 2D) or peak systolic Ca²⁺ (Figure 2E–2F) between the DMSO-treated cells (control) or cells treated with 1 or 5 μmol/L sorafenib for 48 hours. These studies show that high doses of sorafenib can induce myocyte necrosis although there were no significant effects of lower doses on myocyte survival or inotropy.

Myocyte lysates collected 24, 48, or 72 hours after exposure to sorafenib at each dose were used for Western analysis of caspase-3 (Figure 2G), a molecule known to be associated with activation of the apoptotic cell death pathways. No activation of caspase-3 at any time point or at any dose of sorafenib was found. A positive control (+) was included in which caspase-3 was activated in isolated myocytes by exposure to 100 μmol/L H₂O₂. Thus, the cell death observed with sorafenib doses above 10 μmol/L appears to be due to necrotic cell death rather than via apoptosis.

**Decreased Heart Size Observed With Sorafenib Treatment Occurs Secondary to Myocyte Death, With Surviving Myocytes Undergoing Pathological Hypertrophy**

In isolated myocytes in vitro, sorafenib treatment clearly induces necrotic cell death but does not appear to affect
contractile function of surviving myocytes. To examine the effects of sorafenib on myocytes in vivo, myocyte size and general tissue distribution were measured using tissue sections from hearts fixed at 1 or 2 weeks post MI. Representative bright-field micrographs from hearts fixed 2 weeks after surgery and stained with Masson trichrome are displayed in Figure 3A. BioQuant was then performed to determine whether there was any difference between groups in the fraction of eosin+ (red) myocardial tissue out of total myocardial tissue (Figure 3B). There was a decline in the percent of myocyte volume fraction after MI surgery in both sorafenib- and vehicle-treated groups. There was no significant difference in myocyte volume fraction between sorafenib-treated and vehicle-treated animals between either the sham-operated or MI groups although sorafenib treatment was associated with decreased heart size measured both grossly and by echocardiography.

Myocyte cross-sectional area was measured in each group (Figure 3C), and >3400 myocytes were analyzed from 40 animals. MI alone resulted in small increases in myocyte cross-sectional area, consistent with post-MI hypertrophy. Interestingly, sorafenib treatment in sham animals also resulted in increased myocyte cross-sectional area although overall heart size was significantly smaller than in vehicle-treated controls. Sham+Sorafenib myocytes had significantly greater cross-sectional areas than vehicle-treated sham controls, and MI+Sorafenib animals had significantly greater myocyte cross-sectional areas than vehicle-treated MI controls. The combination of MI and sorafenib treatment resulted in the largest myocyte cross-sectional areas of all the groups (400–450 μm²). The most likely mechanism by which sorafenib could increase individual myocyte size while decreasing overall HW would be if a significant number of myocytes were lost. We found that sorafenib causes myocyte necrosis at high doses in vitro (Figure 2), and this is the likely mechanism for the loss of myocyte mass in vivo. These results suggest that sorafenib induces myocyte death and the remaining myocytes undergo pathological hypertrophy, so the percentage of muscle tissue/total myocardium is not different between sorafenib- and vehicle-treated groups (in either sham or MI groups).

**Sorafenib Inhibits Stem Cell Proliferation In Vitro and In Vivo**

Recent data suggest that myocytes that die when the heart is subjected to toxic agents can be replaced by the generation of new cardiac myocytes derived from a cKit+ progenitor cell pool. Sorafenib is known to inhibit the c-kit tyrosine kinase receptor, a receptor that is found on cardiogenic stem cells located in the heart and bone. Thus, sorafenib may exert some inhibitory effect on these cell populations, which could also contribute to the high post-MI mortality observed with sorafenib treatment. Figure 4A shows cultures of isolated cortical bone–derived stem cells or cardiac-derived stem cells after 72 hours of exposure to DMSO (control) or 0.1 to 50 μmol/L sorafenib. Cortical bone–derived stem cells and cardiac-derived stem cells were plated at low densities (75000 cells/well) and were allowed to proliferate to confluency for 72 hours. Both stem cell populations rapidly proliferate in culture. Cell counts were performed at 24, 48, and 72 hours. Growth curves show a potent time- and dose-dependent inhibition of stem cell proliferation after exposure to sorafenib (Figure 4B), and this is reflected in the bright-field images (Figure 4A). Western analysis of total versus activated caspase-3 was performed on cell lysates prepared at each time point to determine whether this cell death was due to activation of apoptosis. We found a time- and dose-dependent activation of caspase-3 with corresponding degradation of total caspase-3 signal (Figure 4C). These data suggest that sorafenib inhibits stem cell proliferation and induces stem cell apoptosis. It is possible that stem cells were lost from culture via apoptosis without an effect of sorafenib on stem cell proliferation. However, cultures exposed to high doses of sorafenib (>10 μmol/L) failed to undergo any increase in cell...
number, suggesting inhibition of stem cell proliferation and stem cell apoptosis are occurring simultaneously. These results suggest that sorafenib may inhibit endogenous cardiac repair by modifying the behavior of cKit+ cardiac progenitor cells.

To assess whether sorafenib treatment inhibited stem cell function in vivo, a 5-bromo-2’-deoxyuridine (BrdU) pulse-labeling strategy was used to identify myocytes and non-myocytes undergoing S-phase DNA replication post MI. This strategy has been previously used by our group to identify newly generated myocytes in the feline heart after ischemic insult secondary to catecholamine overload,46 and other groups have used similar strategies to measure newly generated myocytes in the adult mouse heart at baseline47 or after MI.12,16,48,49 Figure 5 shows representative confocal micrographs from the infarct border zone of hearts treated with vehicle (Figure 5A) or sorafenib (Figure 5B) 1 week after MI and BrdU labeling. Quantitative histology showing the percentage of BrdU+ myocytes (Figure 5C) or nonmyocytes (Figure 5D) demonstrates that sorafenib treatment significantly decreases the percentage of both myocyte and nonmyocyte nuclei that are brightly labeled with BrdU after 1-week post MI in the infarct border zone although no significant difference was seen in viable zones. These findings are consistent with the in vitro data in Figure 4, suggesting that sorafenib treatment potently inhibits post-MI stem cell function in vivo.

**Sorafenib-Mediated Myocyte Loss Is Not Caused by Apoptosis In Vivo**

Sorafenib treatment had little effect on apoptosis of cardiac myocytes or nonmyocytes near the infarct border zone or in distal viable myocardium. To quantify the percent of myocytes and nonmyocytes undergoing apoptosis post MI, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed on myocardial tissues fixed at 1 or 7 days post MI. Figure 6A displays a representative confocal micrograph of a vehicle-treated heart 1 day after MI, with the magnified panel showing a binucleated myocyte with 2 TUNEL+ nuclei and an adjacent TUNEL+ nonmyocyte nucleus. Figure 6B depicts the border zone of a sorafenib-treated heart 1 day after MI, with a TUNEL+ myocyte nucleus magnified at right. MI injury alone is known to increase myocyte apoptosis acutely at the infarct border zone in rodents50,51 as well as humans,52 but sorafenib-treated tissues did not have a significantly greater percentage of TUNEL+ nuclei than vehicle-treated controls at 1 or 7 days post MI. Likewise, there was no significant increase in the percentage of TUNEL+ nonmyocyte nuclei detected at the infarct border zone at 1 or 7 days post MI. Similar analyses were performed on distal viable myocardium >1000 μm from the border zone, and this also demonstrated no significant increase in the number of TUNEL+...
myocyte or nonmyocyte nuclei detected in sorafenib-treated hearts after 1 or 7 days post MI compared with vehicle-treated controls (data not shown). These data support the in vitro findings in Figure 2, which suggest that myocytes are lost primarily by necrosis and not apoptosis.

**Sorafenib Does Not Affect Cardiac Fibrosis**

The effects of sorafenib on isolated adult cardiac fibroblasts and the mouse endothelial fibroblast-1 cell line were examined to determine if sorafenib had any effect on post-MI fibrosis. Both fibroblast cell types were plated at low densities (50,000 cells/well) and were allowed to proliferate for 72 hours in the presence of DMSO (control) or 0.1 to 50 μmol/L sorafenib. In Figure 7A, representative bright-field micrographs of mouse endothelial fibroblast-1 cells after 72 hours of exposure to sorafenib are displayed (similar changes were observed in primary mouse adult cardiac fibroblasts cultures; data not included). Cell counts were performed after 24, 48, or 72 hours in culture (Figure 7B). Sorafenib induced a time- and dose-dependent decrease in proliferation of both populations of fibroblasts at doses greater than 5 μmol/L. Lysates were prepared from samples at each time point, and Western analysis against total and activated caspase-3 was performed to determine whether this inhibition of proliferation was due to activation of the apoptotic pathway. Western analysis of mouse endothelial fibroblast-1 lysates demonstrated a time- and dose-dependent activation of caspase-3 in response to sorafenib treatment (Figure 7C). Adult cardiac fibroblasts exhibited similar activation of caspase-3 (data not included).

The effects of sorafenib on fibrosis in vivo were also examined. The number of mice in this MI model that died prior to euthanasia secondary to rupture of the infarct-related wall was not significantly different between MI animals treated with vehicle or 30 mg/kg/d sorafenib (12.0 versus 9.7%; P=not significant). To examine tissue fibrosis, we analyzed Masson trichrome–stained tissue sections from hearts fixed 2 weeks post MI. The percent of total myocardial tissue that was trichrome+ (blue) was determined (Figure 7D). A representative image showing an area of fibrotic scar tissue near the infarct border zone is displayed in Figure 7E along with the corresponding bioquantification analysis (Figure 7F) in which fibrotic tissue is highlighted in green. MI increased the percentage of fibrotic tissue, which is characteristic of the changes seen during post-MI remodeling. However, there was no significant difference in the percentage of fibrotic tissue between Sham+Vehicle versus Sham+Sorafenib or between MI+Vehicle versus MI+Sorafenib. Thus, while higher doses (> 5 μmol/L) of sorafenib can inhibit
fibroblast proliferation by inducing apoptosis (in vitro), there does not appear to be a significant change in fibrosis associated with sorafenib treatment in vivo.

**Sorafenib Does Not Affect Infarct Size or Post-MI Neovascularization**

The effects of sorafenib on infarct size at 24 hours post MI and 2 weeks post MI were examined. Animals from each MI group were selected at random to undergo euthanasia 24 hours post MI. No significant difference was detected in either area at risk or infarct area between sorafenib- and vehicle-treated MI animals (Online Figure IA and IB). To measure chronic infarct size, tissue sections from hearts fixed 2 weeks post MI were stained with Masson trichrome (Online Figure IC), and the percentage of total myocardial area that was pathologically fibrotic was determined. After 2 weeks, there was no difference in infarct size between the sorafenib- and vehicle-treated MI groups (Online Figure ID).

Sorafenib inhibits receptors for the proangiogenic growth factors VEGF and platelet-derived growth factor, which are both involved in post-MI neovascularization. To determine whether sorafenib treatment affected post-MI blood vessel formation, tissues that were fixed at 1 or 2 weeks post MI were immunostained for α-sarcomeric actin (red) and von Willebrand factor (green), and nuclei were labeled with 4',6-diamidino-2-phenylindole (blue). Representative confocal micrographs of tissues from 2 weeks post MI+Vehicle or MI+Sorafenib are shown in Online Figure IE. Quantitative confocal histology was used to determine the number of von Willebrand factor+ vessel structures per visual field (Online Figure IF). No significant difference was found between the number of von Willebrand factor+ vessels between vehicle- and sorafenib-treated MI animals. Thus, sorafenib treatment did not appear to affect post-MI neovascularization within the 2-week time course of these experiments.

**Treatment With Metoprolol Post MI Prevents Sorafenib-Induced Mortality, Improves Cardiac Function, and Reduces Myocyte Cross-Sectional Area**

After MI, activation of the sympathetic nervous system increases the contractility of surviving myocytes and allows the heart to pump sufficient blood to support systemic blood pressure. However, persistent and excessive sympathetic signaling might provoke the damaging effects of sorafenib and contribute to the excess mortality of MI in sorafenib-treated animals. To test this idea, an additional group of mice underwent MI+30 mg/kg/d IP sorafenib dosing (n=20), and after MI, these mice received 20 mg/kg/d IP metoprolol. Metoprolol treatment improved 2-week survival post MI to 38.9%, a level not significantly different from vehicle-treated MI controls (Figure 8). These findings suggest that catecholamines contribute to the increased mortality after MI in sorafenib-treated animals.
Additionally, metoprolol treatment improved post-MI ejection fraction and fraction shortening relative to sorafenib-treated controls (Figure 8B and 8C). Metoprolol also attenuated remodeling relative to vehicle-treated MI controls, as has been widely reported in the literature. At the cellular level, myocytes from metoprolol treated hearts did not have significantly larger cross-sectional areas than vehicle-treated or sham-operated animals and were significantly smaller than myocytes treated with sorafenib alone (Online Figure II), suggesting that metoprolol prevented the pathological hypertrophy of myocytes observed after sorafenib treatment. Considering that cardiac volumes measured by echocardiography and gross HWs of Sorafenib+Metoprolol treated animals were significantly larger than mice treated with sorafenib alone (Figure 8C), the smaller myocyte size in the presence of larger overall heart size suggests that metoprolol treatment prevented sorafenib-induced myocyte death.

Discussion

Our studies show that sorafenib induces myocyte death, even in the absence of cardiac injury, and when administered in the presence of MI, sorafenib dramatically increases mortality (Figure 1A). This myocyte loss appears to be due to necrosis (possibly a programmed form of necrosis) and not apoptosis (Figures 2G and 6), which is consistent with our previously published reports on imatinib-induced cardiac injury. Myocytes that survive the sorafenib treatment undergo pathological hypertrophy (Figure 3) to maintain contractile mass, and cardiac function remained relatively unchanged (Figure 1C). As surviving myocytes increase in size, they could reach a size at which basic cell metabolism is significantly disrupted, resulting in necrotic cell death. Ultimately, recurrent and widespread myocyte loss leads to hemodynamic instability that can culminate in death. Sorafenib-induced cardiac injury does not appear to be secondary to any direct effects of the drug on cardiac inotropy because sorafenib lacked any direct negative inotropic effects on isolated myocytes (Figure 2C–2F).

In combination with myocyte necrosis, sorafenib also potently induces stem cell apoptosis and inhibits stem cell proliferation in vitro (Figure 4) and in vivo (Figure 5). These effects may inhibit the generation of new cardiac myocytes after MI, further exacerbating cardiac dysfunction. These findings are consistent with previous research on c-kit-deficient mice, which have markedly reduced post-MI repair and survival. The authors of these studies attributed this decreased survival mostly to failed recruitment of stem cells from the bone marrow specifically, but our data show that c-kit antagonism potently inhibits all pools of cardiogenic stem cells in the body, including those residing in the heart and cortical bone (Figures 4 and 5). Sorafenib potently diminished in vivo BrdU labeling of myocytes and nonmyocytes, further suggesting that cardiac repair and new myocyte formation mediated by c-kit+ stem cells is inhibited by sorafenib injury.
Similar observations have been reported in a mouse model of diabetic cardiomyopathy, which similarly showed decreased cardiac mass and volume secondary to myocardial cell loss with surviving myocytes undergoing hypertrophy. In this model, cardiac-derived stem cells were lost by a 4-fold higher rate of apoptotic death than in myocytes, which were shown to die via necrosis. The authors speculated that hyperglycemia increased local levels of radical free oxygen species in the myocardium and that the differential mechanisms of death between cardiac-derived stem cells and myocytes were due to differing susceptibilities of each cell type to radical free oxygen species. The similarities to our mouse model are striking, suggesting that sorafenib injury might increase the levels of local myocardial radical free oxygen species, which could explain the differential mechanisms of cell death observed in Figures 2 and 4.

Sorafenib-induced cardiotoxicity does not appear to be related to sorafanib-mediated inhibition of RAF1. Conditional, cardiac-specific RAF1 knockout resulted in cardiac dilation with myocyte apoptosis and increased cardiac fibrosis. Our results show that sorafenib induced myocyte necrosis and not apoptosis (Figures 2G and 6) and resulted in decreased heart size rather than dilation (Figure 1C). Necrotic myocyte death and decreased heart size were also observed in our previously published report on imatinib cardiotoxicity, suggesting that these anticancer drugs might work through a common pathway. Sorafenib inhibited the growth of both primary adult cardiac fibroblasts and mouse endothelial fibroblast-1 cells in vitro but did not have any effect on the degree of in vivo fibrosis over the time frame of the present experiments (Figure 5). However, the majority of the mortality observed in our MI model occurred before 2 weeks post MI, which is still relatively early in the post-MI remodeling process. Thus, it is possible that had our mice survived longer, we may have observed a greater degree of inhibition of fibrosis in vivo. However, our in vitro results suggest that we would not expect any increase in fibrosis as was seen in the conditional RAF1 knockout mouse. It does not appear that RAF1 is involved in sorafenib-mediated cardiotoxicity.

Another proposed mechanism of multikinase inhibitor cardiotoxicity involves antagonism of VEGF receptor and platelet-derived growth factor receptor and inhibition of post-MI neovascularization. The heart normally has a robust angiogenic response to ischemic injury to reestablish a supply of oxygen and nutrients to the ischemic tissue. Our data would suggest that VEGF receptor and platelet-derived growth factor receptor antagonism by sorafenib does not play a major role in the increased mortality observed in this
model because after 1 and 2 weeks post MI, no significant difference in blood vessel density could be detected at the infarct border zone between vehicle- and sorafenib-treated MI mice (Figure 6). However, we have previously shown that the most robust post-MI neovascularization in our animal model occurs after 6 weeks post MI,14 so it is possible that had the mice survived longer than 2 weeks, we may have observed some decrease in neovascularization in sorafenib-treated mice. However, the major mechanism(s) of premature death occurs without any changes in neovascularization and is related to the loss of myocytes and failure of stem cell–mediated repair of the heart.

The increased mortality observed after MI in sorafenib-treated animals can be completely reversed by concomitant administration of the β1-adrenergic antagonist metoprolol (Figure 8). The exact mechanism of this improvement in survival and cardiac structure and function was not completely elucidated. However, excessive adrenergic activity in the failing heart is responsible for critical aspects of heart failure progression60 and myocyte death signaling.61–63 Our data suggest that metoprolol eliminates sorafenib-mediated myocyte death to improve cardiac function and reduce pathological hypertrophy and loss of myocytes. Sorafenib appears to require persistent catecholamine signaling to induce excess mortality after MI. We speculate that blocking the increased autonomic outflow to the heart post MI reduces myocyte loss by blunting Ca2+-overload-induced myocyte necrosis.

**Conclusions**

Tyrosine kinase inhibitors such as sorafenib have revolutionized cancer therapy and saved many lives. Sorafenib continues to be a mainstay in the clinical treatment of RCC, and its use is now being expanded to treat other cancers including hepatocellular carcinoma and melanoma.7 We are not advocating against its future use. However, especially in patients with a known history of cardiac comorbidities, a more thorough cardiac work-up should be considered prior to the use of this drug. Furthermore, the presence of these comorbidities demands close follow-up of patients receiving this agent. There is little availability of baseline cardiac function data in patients who have received this drug in the past because studies such as echocardiography are rarely warranted in patients with renal carcinoma. However, there is increasing evidence that these sorts of studies should be used to identify patients who might be susceptible to the adverse cardiotoxic features of this drug. Our results would suggest that any ischemic damage or myocardial cell loss is likely to be profoundly exacerbated by sorafenib. Additionally, in any patients who are already at high risk of MI (those with ischemic cardiomyopathy, coronary artery disease, and chronic hypertension), physicians should consider protective adjunct therapies. Finally, our findings suggest that β1-adrenergic antagonists, commonly used heart failure therapeutic agents, could protect patients at risk of sorafenib-mediated cardiotoxicity.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is Known?**

- Kinase inhibitors specifically targeting receptors that become constitutively activated in cancer cells while sparing healthy host cells and reducing side effects compared with traditional chemotherapeutics.
- The multikinase inhibitor sorafenib, which is used for the treatment of solid tumors such as renal cell carcinoma, can cause severe cardiac dysfunction in human subjects through unknown mechanisms.

**What New Information Does This Article Contribute?**

- Sorafenib induces loss of myocytes by necrosis, without affecting myocyte contractility or overall cardiac function.
- Sorafenib treatment induces apoptotic death of stem cells in the heart and bone, which decreases myocyte turnover at the infarct border zone and reduces endogenous repair of the heart post–myocardial infarction, further exacerbating cardiac dysfunction after myocyte loss.
- The cardiotoxic effects of sorafenib are reversed by concomitant administration of the selective β₁-adrenergic antagonist metoprolol, which reversed the increased mortality caused by sorafenib treatment and prevented sorafenib-induced myocyte loss.

Kinase inhibitors, including sorafenib, are more selective, with a potential of relatively few systemic side effects. Although patients treated with kinase inhibitor live longer, recent clinical evidence has begun to document their cardiotoxicity. In this study, we describe how sorafenib increases mortality after myocardial infarction in a mouse model. This increased mortality is caused by sorafenib-induced necrotic death of myocytes. The myocyte loss is exacerbated by sorafenib-mediated apoptosis of cardiogenic stem cells, which prevents normal myocyte turnover and inhibits endogenous repair at the infarct border zone. Sorafenib cardiotoxicity was ameliorated by treatment with metoprolol, which improved post–myocardial infarction survival of sorafenib-treated animals and reduced the degree of myocyte loss. This study suggests that patients with renal cell carcinoma could prophylactically take metoprolol to reduce the risk of cardiotoxicity while remaining on sorafenib to treat their malignancy.
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Jason M. Duran, Catherine A. Makarewich, Danielle Trappanese, Polina Gross, Sharmeen Husain, Jonathan Dunn, Hind Lal, Thomas E. Sharp, Timothy Starosta, Ronald J. Vagnozzi, Remus M. Berretta, Mary Barbe, DaoHai Yu, Erhe Gao, Hajime Kubo, Thomas Force and Steven R. Houser

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Supplemental Material

**Online Materials and Methods**

**Sorafenib and Metoprolol Dosing.** For all *in vivo* experiments, doses of 30 mg/kg/d sorafenib were used based on preclinical mouse models that demonstrated efficacy of this dose at inhibiting cancer growth in mouse models. Preclinical trials showed that doses of 30 mg/kg/d in mice effectively reduced the proliferation of VEGF-dependent tumors in mice.\(^1,2\) This dose blocked growth and proliferation of both RCC\(^3\) and hepatocellular carcinoma (HCC) tumors\(^4\) *in vivo* in mouse models. Doses below 30 mg/kg/d were not effective at inhibiting tumor growth in the same mouse models.\(^3\) In humans, phase II and III clinical trials have demonstrated that 400 mg twice daily (800 mg/d) is the most effective dose for these same malignancies (HCC and RCC), and this dose achieves a plasma concentration of 2-6 gm/L\(^5\) or about 1-3 mM\(^6\). Plasma levels of sorafenib were attained by liquid chromatography/mass spectrometry\(^7\). These same clinical trials have also reported cardiotoxicity at this same dose. For our *in vitro* experiments, 0.1-50 uM sorafenib was used. These *in vitro* doses encompass the range of those used in preclinical studies during the development of sorafenib\(^1\). For *in vivo* metoprolol dosing, animals received 20 mg/kg/d metoprolol tartrate (Sigma-Aldrich; St. Louis, MO). This is a similar IP dose of metoprolol that has been used in previously published mouse models to affect airway reactivity\(^8\) and reduce post-MI LV dysfunction in a mouse MI model.\(^9\)

**Cell Isolation and Culture.** Adult feline left ventricular myocytes were isolated and cultured by perfusion digestion of the heart and fractional shortening and calcium transients were measured as previously described.\(^10-12\) Bone-derived and cardiac-derived stem cells were isolated from C57BL/6 mice (The Jackson Laboratory; Bar Harbor, ME) and cultured as previously described.\(^12\) Adult cardiac fibroblasts were
isolated from C57BL/6 mice using a modified previously described protocol.\textsuperscript{13, 14} Hearts were isolated from mice and aortas were cannulated and perfusion digested with collagenase-containing tyrodes solution. After digestion, the ventricles were trimmed free and minced and filtered through sterile nylon mesh. Isolated cells were plated in Iscove’s Modified Dulbeccos Medium (Sigma-Aldrich; St. Louis, MO) containing 10% bovine calf serum and penicillin-streptomycin-glutamine (Gibco Life Technologies; Carlsbad, CA). After a 2-hour incubation period at 37°C in an atmosphere at 5% CO\textsubscript{2}-95% air, the unattached cells were removed and the attached cells (mostly fibroblasts) were grown. All experiments were performed using cells of the first passage. Mouse endothelial fibroblasts (MEF-1 cells) were purchased from the American Type Culture Collection (ATCC #CRL-2214; Manassas, VA) and grown in Iscove’s Modified Dulbeccos Medium (Sigma-Aldrich; St. Louis, MO) containing 10% bovine calf serum and penicillin-streptomycin-glutamine (Gibco Life Technologies; Carlsbad, CA). For all in vitro dosing experiments, sorafenib p-toluenesulfonate salt (LC Laboratories; Woburn, MA) was dissolved in DMSO with sonication and then diluted to the desired concentration in the appropriate cell culture media. For all controls, DMSO was added in equivalent volume to that in the highest dose of sorafenib.

\textit{Western Analysis.} Lysates from myocytes, stem cells, or fibroblasts were prepared and analyzed using Western analysis as previously published.\textsuperscript{10, 12, 15, 16} The following primary antibodies were used: total caspase-3 (Cell Signaling #9662, Beverly, MA), activated caspase-3 (Cell Signaling #9661; Beverly, MA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AbD Serotec #MCA4739G; Kidlington, UK). The following secondary antibodies were used: donkey anti-rabbit-HRP (GE#NA934V) and sheep anti-mouse-HRP (GE#NA931V) purchased from GE Healthcare (Little Chalfont, UK).

Animals, Anesthesia, and In vivo Procedures. All animal procedures were
approved by the Temple University School of Medicine Institutional Animal Care and Use Committee. Anesthesia was induced and maintained using isoflurane (Butler Shein Animal Health; Dublin, Ohio) following previously published protocols.\textsuperscript{12} All animals used for \textit{in vivo} studies were 12-week-old male C57BL/6 mice (The Jackson Laboratory; Bar Harbor, ME). Adult feline left ventricular myocytes were isolated as previously described \textsuperscript{10, 17, 18}. Isolated myocytes were washed with serum-free Medium 199 (Sigma-Aldrich; St. Louis, MO) supplemented with penicillin-streptomycin-glutamine (Gibco Life Technologies; Carlsbad, CA) and cultured on plates coated with laminin (BD Bioscience; Franklin Lakes, NJ).

For \textit{in vivo} dosing, 25 mg/mL sorafenib p-toluenesulfonate salt (LC Laboratories; Woburn, MA) stock solution was prepared in a 1:1 mixture of Cremaphor EL (Sigma-Aldrich; Carlsbad, CA) and 95% ethanol as previously described.\textsuperscript{19} Fresh stock solution was made every 3 days. For intraperitoneal (IP) injection, sorafenib stock solution was diluted 1:5 in sterile normal saline. Animals received 30 or 40 mg/kg/day IP sorafenib every day for 3 weeks. After 1 week of dosing, animals underwent sham operation or myocardial infarction (MI) by permanent occlusion of the left anterior descending coronary artery as previously described.\textsuperscript{12, 20} Animals were allowed to recover after surgery, then osmotic minipumps (Alzet; Cupertino, CA) containing 78.125 mg/mL 5-bromo-2’-deoxyuridine (BrdU, Sigma-Aldrich; Carlsbad, CA) in 50/50% DMSO/ddH\textsubscript{2}O were implanted subcutaneously between the two scapulae to deliver a continuous infusion of BrdU for 7 days. After 7 days, all pumps were removed. Some animals were selected at random from each group and sacrificed for acute area at risk (AAR) measurement using 2% Evan’s blue dye perfusion and ischemic area (IA) analysis using triphenyltetrazolium chloride staining at 24 hours post-MI as previously described.\textsuperscript{12} All mice underwent non-invasive transthoracic echocardiography following previously published protocols\textsuperscript{12, 16, 21} at baseline prior to sorafenib dosing, at 1 week after sorafenib
dosing but before sham or MI surgery, and at 1 and 2 weeks after surgery. Cardiac function and dimensions were analyzed in a randomized, blinded fashion from 2-dimensional M-mode as previously described.\textsuperscript{12, 16, 21}

A total of 154 mice were used for this study. All animals were assigned a sacrifice date prior to operation to avoid selection bias. Of the sham-operated animals that received vehicle dosing (n=16), 5 animals were sacrificed at 1 week post-surgery and 11 animals were sacrificed at 2 weeks post-surgery. The hearts from all animals were perfusion-fixed with 10% formalin and paraffin embedded as previously described.\textsuperscript{12, 16, 21} Of the animals that underwent sham-operation with 30-mg/kg/day sorafenib dosing (n=10), 5 were sacrificed at 1 week and the remaining 5 were sacrificed at 2 weeks after surgery. All hearts were fixed and embedded at each time point and no animals were lost prior to sacrifice.

Of animals that underwent induced MI with vehicle dosing (n=33), 5 animals were randomly selected for sacrifice at 24 hours post-MI for infarct size analysis and 3 animals were sacrificed and their hearts were perfusion fixed and paraffin embedded for histology. These 8 animals were not included in Kaplan-Meier survival analysis. Another 11 animals died in the first week post-MI and 14 survived to undergo 1-week post-MI echocardiography. Of the 14 survivors, 7 were sacrificed at 1-week post-MI and their hearts were fixed and embedded for histology. Of the remaining 7 animals, 2 died during the second week post-MI and 5 animals survived to undergo 2-week post-MI echocardiography. These 5 surviving animals were sacrificed and their hearts were fixed and embedded for histology.

Of the animals that underwent induced MI with 30 mg/kg/day sorafenib dosing (n=57), 5 animals were randomly selected for sacrifice at 24 hours post-MI for infarct size analysis and 3 were sacrificed and their hearts were fixed and embedded for histology. These animals were not included in Kaplan-Meier analysis. Another 31
animals died in the first week post-MI and 18 animals survived to undergo 1-week post-MI echocardiography. Of these 18 animals, 4 were sacrificed at 1-week post-MI and their hearts were fixed and embedded for histology. Of the remaining 14 animals, 10 died during the second week post MI and 4 animals survived to undergo 2-week post-MI echocardiography. These 4 surviving animals were sacrificed and their hearts were fixed and embedded for histology.

Of the animals that underwent induced MI with 40 mg/kg/day sorafenib dosing (n=20), 14 animals died in the first week post-MI and 6 survived to undergo 1 week post-MI echocardiography. Of these 6 animals, 2 were sacrificed at 1-week post-MI and their hearts were perfusion fixed and paraffin-embedded. Of the remaining 4 animals, 3 died in the second week post-MI and 1 animal survived for 2-week post-MI echocardiography. The last remaining animal was sacrificed at 2 weeks post-MI and the heart was fixed and embedded for histology. Because only one survivor was left in this group after two weeks, there was insufficient sample size to conduct statistical analysis, so the echocardiography data for this group was not included in the manuscript. Similar changes in cardiac function and ventricular volumes were observed in this group as those that received MI+30 mg/kg/d sorafenib.

An additional group of animals underwent induced MI with 30 mg/kg/day sorafenib dosing (n=19). After the MI procedure, these animals received 20 mg/kg/day IP metoprolol for the remaining two weeks of the study. All animals in this group were included in Kaplan-Meier analysis. 8 animals died in the first week post-MI and 10 survived to undergo 1-week post-MI echocardiography. Of these 10 animals, 7 survived to undergo echocardiography at 2 weeks post-MI and their hearts were perfusion fixed and paraffin-embedded for histology.

_Tissue Processing and Histology._ Animals were sacrificed at 1 or 2 weeks after surgery and their hearts were removed under anesthesia, perfusion fixed, and paraffin
embedded for histology following previously published protocols. Samples were stained with Masson’s trichrome (Sigma-Aldrich; St. Louis, MO) for myocyte cross-sectional area, BioQuant analysis, and 2-week infarct size analysis. To determine the percent eosin+ (red tissue) and the percent trichrome+ (blue tissue) out of the total myocardial area, BioQuant analysis was performed as previously described. Myocyte cross-sectional area was measured on Masson’s trichrome-stained tissue sections using NIH Image J software. The number of myocytes analyzed per group is shown within each bar on the charts in Figure 3C and Supplemental Figure S2. Myocytes from all regions of the heart were analyzed (near and distant to the infarct zone) to generate a representative average cross-sectional area for each group. For Figure 8 and Online Figure II, the four control groups (Sham+Vehicle, Sham+Sorafenib, MI+Vehicle and MI+Sorafenib) are the same controls used in Figures 1 and 3, respectively. Because of the high mortality observed in this animal model (almost 90% 2 week mortality in the MI+30 mg/kg/d sorafenib group), the same control groups were used for statistical comparison to MI+Sorafenib+Metoprolol group in order to reduce the overall number of animals used for the study.

Immunostaining was performed as previously described to quantify vascular density at the infarct border zone, BrdU labeling, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL). The following primary antibodies were used: mouse IgM anti-α-sarcomeric actin (Sigma-Aldrich #A2172; St. Louis, MO), rabbit anti-von Willebrand factor (Abcam #ab6994; Cambridge, MA), mouse IgG anti-BrdU (11 296 736 001, Roche; Basel, Switzerland), and mouse IgG anti-sarcomeric tropomyosin (Sigma-Aldrich #T9283; St Louis, MO). The DeadEnd Fluorometric TUNEL system was used to label apoptotic nuclei (Promega #G3250; Madison, WI). The following secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and used for detection of
primary antibodies as follows: rhodamine red-X donkey anti-Mouse IgM (715-295-020) was used to detect α-sarcomeric actin, FITC donkey anti-rabbit IgG (711-095-152) was used to detect von Willebrand factor, FITC donkey anti-mouse IgG (715-095-151) was used to detect BrdU, and Rhodamine Red-X donkey anti-mouse IgG (715-295-150) was used to detect tropomyosin. For all immunostains, nuclei were labeled with 4’,6-diamidino-2-phenylindole (DAPI, Millipore; Billerica, MA). Confocal micrographs of all immunostains were acquired using a Nikon Eclipse T1 confocal microscope (Nikon Inc.; Melville, NY).

For quantification of BrdU labeling, the percentage of BrdU-labeled myocyte and non-myocyte nuclei was quantified per tissue cross-section either at the infarct border zone or in distant viable zones. A nucleus was only counted as “BrdU+” if BrdU signal (green) was colocalized with the nuclear marker DAPI (blue) to control for non-specific antibody labeling. All photography and analysis was performed using at least 40X optical magnification, and BrdU-positivity was confirmed live on the confocal microscope by scanning each nucleus in question along the Z-axis. Border zone was identified by the degradation of α-sarcomeric signal, and cells were classified as residing in the border zone if they were located within 500 µm (two 40X visual fields) in either direction of the degraded actin boundary. Viable zones were classified as being at least 1000 µm away from the border zone (typically in the non-infarcted posterior wall of the left ventricle, or in the intraventricular septum). Over 210,000 total nuclei (including almost 25,000 myocyte nuclei) were analyzed from 8 mice sacrificed at 1 week post-MI that had been treated with either sorafenib or vehicle.

For quantification of TUNEL staining, the percentage of TUNEL+ myocyte and non-myocyte nuclei was similarly quantified in each tissue cross-section either at the infarct border zone or in distant viable zones. As with BrdU analysis, a nucleus was only counted as “TUNEL+” if the TUNEL signal (green) colocalized with the nuclear marker
DAPI (blue) and TUNEL-positivity was confirmed live on the confocal microscope by scanning each nucleus along the Z-axis. The border zones and viable zones were defined as stated previously. Over 40,000 nuclei, including over 5,000 myocyte nuclei, were analyzed from 12 mice sacrificed at 1 or 7 days post-MI from mice treated with either vehicle or sorafenib.

**Statistical Analysis.** The student’s T-test was used to analyze infarct size data (24 hour AAR and IA, and 2 week infarct area) and BrdU labeling. One-way analysis of variance (ANOVA) was used to analyze isolated myocyte physiology data, *in vivo* myocyte nuclei counts and cross-sectional area, BioQuant analysis of muscle and fibrotic area, blood vessel counts and TUNEL analysis. For stem cell and fibroblast proliferation experiments, ANOVA was used to analyze data at the 72-hour time point, and for myocyte survival counts ANOVA was used to analyze the data at the 12, 24, and 36-hour time points. To analyze echocardiography data over time at -1, 0, 1 and 2 weeks, the profiles and comparisons of the five treatment groups were made using the mixed-effects modeling and the Kruskal-Wallis test was used to determine statistical significance when the parametric model assumptions are invalid. The post-MI survival data were analyzed using the Kaplan-Meier product-limit approach and comparisons between the treatment groups were made via the log-rank test. For all analyses, a *p*-value of < 0.05 was considered statistically significant.
References


10. Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Berretta RM, Rizzo V, Molkentin JD and Houser SR. A caveolae-targeted L-type Ca(2)+ channel antagonist


Online Figure I: Sorafenib does not effect infarct size or post-MI angiogenesis. Hearts from animals sacrificed at 24 hours post-MI were perfused with Evan’s blue and stained with triphenyltetrazolium chloride, and brightfield images were acquired (A) to measure area at risk (AAR) and infarct area (IA), respectively (B). Hearts fixed at 2 weeks post-MI were stained with Masson’s trichrome (C) and the percent of total myocardial surface area that was pathologically infarcted was calculated (D). Other sections from hearts fixed 2 weeks post-MI (E) were immunostained for α-sarcomeric actin (red) and von Willebrand factor (vWF, green), and nuclei are labeled with DAPI (blue). Scale bars = 200 µm. The number of vWF+ vessels per visual field was quantified (F).

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<thead>
<tr>
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<th>MI+Vehicle</th>
<th>MI+30mg/kg/d SF</th>
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<tr>
<td><strong>Percent (%)</strong></td>
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<td><strong>vWF+ Vessels/Field</strong></td>
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<tr>
<td>1 Week</td>
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<td>2 Weeks</td>
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**NS** = Not Significant (p > 0.05)
Online Figure II: Metoprolol treatment reduces pathologic hypertrophy of myocytes secondary to sorafenib-mediated myocyte loss. A) Representative brightfield micrographs of Masson’s trichrome stained sections of mouse tissues 2 weeks after MI with vehicle treatment, 30 mg/kg/d sorafenib treatment alone, or sorafenib+20 mg/kg/day metoprolol. Scale bars = 20 µM. B) Average cross-sectional areas of myocytes from each group. * = p < 0.05