A Novel Preclinical Strategy for Identifying Cardiotoxic Kinase Inhibitors and Mechanisms of Cardiotoxicity

Hui Cheng, Gabor Kari, Adam P. Dicker, Ulrich Rodeck, Walter J. Koch, Thomas Force

Rationale: Despite intense interest in strategies to predict which kinase inhibitor (KI) cancer therapeutics may be associated with cardiotoxicity, current approaches are inadequate. Sorafenib is a KI of concern because it inhibits growth factor receptors and Raf-1/B-Raf, kinases that are upstream of extracellular signal-regulated kinases (ERKs) and signal cardiomyocyte survival in the setting of stress.

Objectives: To explore the potential use of zebrafish as a preclinical model to predict cardiotoxicity and to determine whether sorafenib has associated cardiotoxicity, and, if so, define the mechanisms.

Methods and Results: We find that the zebrafish model is readily able to discriminate a KI with little or no cardiotoxicity (gefitinib) from one with demonstrated cardiotoxicity (sunitinib). Sorafenib, like sunitinib, leads to cardiomyocyte apoptosis, a reduction in total myocyte number per heart, contractile dysfunction, and ventricular dilatation in zebrafish. In cultured rat cardiomyocytes, sorafenib induces cell death. This can be rescued by adenovirus-mediated gene transfer of constitutively active MEK1, which restores ERK activity even in the presence of sorafenib. Whereas growth factor–induced activation of ERKs requires Raf, α-adrenergic agonist-induced activation of ERKs does not require it. Consequently, activation of α-adrenergic signaling markedly decreases sorafenib-induced cell death. Consistent with these in vitro data, inhibition of α-adrenergic signaling with the receptor antagonist prazosin worsens sorafenib-induced cardiomyopathy in zebrafish.

Conclusions: Zebrafish may be a valuable preclinical tool to predict cardiotoxicity. The α-adrenergic signaling pathway is an important modulator of sorafenib cardiotoxicity in vitro and in vivo and appears to act through a here-to-fore unrecognized signaling pathway downstream of α-adrenergic activation that bypasses Raf to activate ERKs. (Circ Res. 2011;109:00-00.)

Key Words: zebrafish □ kinase inhibitors □ cancer □ cardiotoxicity □ ERK

Cardiotoxicity of cancer therapeutics has become a significant problem and probably will continue to be so with the explosion in drugs targeting kinases that are mutated or overexpressed in cancer. Cardiotoxicity with these agents will continue to plague drug development until reliable preclinical screening strategies are developed. Unfortunately, at this point, there are few if any preclinical models that can accurately predict cardiotoxicity, leading on occasion to unfortunate surprises.1,2 Cell lines, which are typically noncontractile and glycolytic, bear little relationship to cardiomyocytes and do not appear to be reliable models for predicting cardiotoxicity. In the future, induced pluripotent stem cell–derived cardiomyocytes from patients with demonstrated cardiotoxicity might provide insights into mechanisms of cardiotoxicity, but this is not a practical screening approach at the present time. Primary cardiomyocytes have been used successfully to examine mechanisms of toxicity, but the general consensus is that a reliable in vivo model is needed. Rodents have been used for this purpose but can be insensitive, particularly when end points are based on measurements of left ventricular contractile function.2 This may be due, at least in part, to the ability of rodents to compensate for loss of myocytes by recruiting compensatory mechanisms, and to the fact that rodents, unlike the typical cancer patient, have no comorbidities (eg, coronary artery disease or hypertension). Indeed, we have found that even with agents known to have associated cardiotoxicity (eg, sunitinib), left ventricular (LV) function can be maintained in rodents, even in the setting of an additional stressor (ie, moderate hypertension).2,3 Transmission electron microscope (TEM) may be the most sensitive technique but quantification of abnormalities on TEM is very difficult. Over the past decade, the zebrafish (Danio rerio) has gained popularity as a model organism for human disease research. Zebrafish possess several advantages over other models for cardiovascular research.4,5 Most importantly, they have a closed cardiovascular system that can readily be
Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>Ki</td>
<td>kinase inhibitor</td>
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<tr>
<td>LV</td>
<td>left ventricular</td>
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<tr>
<td>LVEF</td>
<td>left ventricular ejection fraction</td>
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<tr>
<td>MAPK</td>
<td>MAP kinase, mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/extracellular signal-regulated kinase kinase</td>
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<tr>
<td>Raf</td>
<td>rapidly accelerated fibrosarcoma</td>
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Studied during development because the fish are transparent. In addition, techniques for detailed and quantitative phenotyping of zebrafish heart mutants are available. Because zebrafish can survive in the absence of cardiac output and in the presence of major vascular defects for several days, abnormalities can be studied that would be rapidly fatal in mammals. Finally, zebrafish may be useful for cardiovascular drug discovery because the fish are readily permeable to small molecule drugs when they are added to incubation medium.6–7

Given the above, we asked whether zebrafish might serve as a model to predict cardiotoxicity of small molecule kinase inhibitors. The zebrafish kinome is very similar to human, especially in the ATP pocket, where most inhibitors interact with the kinase.8 We used (1) morphometric analysis, including evidence of pericardial edema, a marker of cardiac dysfunction in fish embryos, (2) staining of whole fish for cardiomyocyte apoptosis, (3) determination of total cardiomyocyte number per heart, using a fish in which cardiomyocytes are readily identified in vivo, and (4) videomicroscopy to quantify wall thickness and contractile function of the fish. We used 3 kinase inhibitors (KIs): 1 with well-documented inhibitors. The zebrafish kinome is very similar to human, especially in the ATP pocket, where most inhibitors interact with the kinase.8 We used (1) morphometric analysis, including evidence of pericardial edema, a marker of cardiac dysfunction in fish embryos, (2) staining of whole fish for cardiomyocyte apoptosis, (3) determination of total cardiomyocyte number per heart, using a fish in which cardiomyocytes are readily identified in vivo, and (4) videomicroscopy to quantify wall thickness and contractile function of the fish. We used 3 kinase inhibitors (KIs): 1 with well-documented cardiotoxicity (sunitinib).2,9,10 1 with minimal to no signal for LV dysfunction or heart failure (gefitinib), and 1 with questionable cardiotoxicity (sorafenib). To our knowledge, there are only 2 studies that have examined sorafenib cardiotoxicity. One reported significant drug-related cardiac abnormalities but the vast majority of these were ECG abnormalities or minor increases in cardiac injury bio-markers (creatine kinase-MB or troponin T).10 Left ventricular ejection fraction (LVEF) was found to be below the lower limit of normal at the time of “cardiac events” in 21.4% of patients but the significance of this is entirely unclear because baseline LVEF was not determined. The only study that examined baseline and serial LVEF determinations in patients on sorafenib reported that mean LVEF declined only 0.8–1.2 EF percent.11 The authors concluded that the effects on LVEF were modest and were unlikely to be of clinical significance, but 13% of patients had significant declines in EF (≥10 EF points).

We ask whether the zebrafish model can (1) discriminate cardiotoxicity versus none (comparing sunitinib versus gefitinib) and (2) predict whether sorafenib is likely to have significant cardiotoxicity. Our findings demonstrate feasibility and support the ability of the model to predict cardiotoxicity. We support these studies with studies in isolated, contracting neonatal rat cardiomyocytes as a confirmatory approach. We then identify inhibition of Raf-1/B-Raf as a key mechanism of cardiotoxicity of sorafenib by partially rescuing sorafenib-induced cardiomyocyte death with adenovirus-mediated gene transfer of a constitutively active MEK1, a kinase immediately downstream of Raf-1/B-Raf and upstream of extracellular signal-regulated kinase (ERK1/2) in the ERK/MAP kinase cascade. We then identify a novel Raf-independent pathway from α-adrenergic receptors to ERK activation that appears to play a role in limiting cardiotoxicity of sorafenib. Finally, we demonstrate the importance of this α-adrenergic pathway in the fish in vivo in limiting sorafenib cardiotoxicity. We believe these studies identify a strategy to screen for cardiotoxicity and describe an approach that can be used to both confirm cardiotoxicity in mammalian cardiomyocytes and to identify the key pathways mediating cardiotoxicity of specific agents in NRVMs in culture and in fish in vivo.

Methods

Zebrafish use and handling at the Thomas Jefferson University Zebrafish Facility was approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University. Wild-type or transgenic adult fish lines were mated in embryo collection tanks. Viable embryos were washed with embryo medium (EM) and sorted (30 embryos per 60-mm dish in 10 mL EM) at the 1- to 2-cell developmental stage (approximately 0.5–1 hour after fertilization [hpf]), and then were maintained under normoxic conditions at 28.5°C. EM was changed after dechorionation at 24–48 hpf and again at 72–96 hpf. For zebrafish to be examined by videomicroscopy or fluorescence microscopy, embryo medium containing 1-phenyl-2-thiourea (PTU, 50 μmol/L) was used to suppress pigmentmentation. Zebrafish were treated with the various KIs at the concentrations and for the times noted in the Figure legends. Unless otherwise noted, treatment occurred at 2 dpf. Toxicity analyses were conducted by monitoring survival and morphology of zebrafish for up to 7 dpf.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Sorafenib and Sunitinib Are Cardiotoxic in the Zebrafish Model

To examine whether sorafenib or sunitinib induced cardiotoxicity in zebrafish, we treated zebrafish at 2dpf with various concentrations of sorafenib, sunitinib and, as negative controls, gefitinib or vehicle. Treatment with sorafenib and sunitinib but not gefitinib increased mortality in the fish but only at a high concentration (5 μmol/L; Figure 1A). Both agents (but not gefitinib) at 5 μmol/L also induced noticeable body malformations that included a curved body shape and uninflated swim bladder. Pericardial edema, a marker of cardiac dysfunction in the fish, was particularly pronounced (Online Figure I).

Based on previous reports examining pharmacokinetics in phase I clinical trials,11,12 the maximum plasma concentration of sorafenib after a 28-day cycle in patients receiving 400 mg 2 times per day was 8.5 μmol/L and the trough concentration was 6.4 μmol/L. As for sunitinib, trough levels have been...
reported to be in the range of 125–250 nmol/L. However, given the large volume of distribution of sunitinib (≈2230 L; www.pfizer.com/files/products/uspi_sutent.pdf), the tissue levels are predicted to be in the 1–3 μmol/L range. Thus, in the following experiments we chose what we believed to be a conservative concentration of 0.5 μmol/L. At this concentration we did not observe any obvious body malformations or pericardial edema in the fish (Figure 1B), nor did we observe any malformations of the vasculature in KI-treated TG:VEGFR2-GRCFP transgenic zebrafish (Online Figure II).

We next used videomicroscopy to quantify cardiac function in the drug-treated zebrafish heart. A representative cardiac image at end-systole in a fish at 5 dpf that had been treated with sorafenib (0.5 μmol/L) at 2 dpf is shown in Figure 2. We quantified end-diastolic dimension (EDD) and end-systolic dimension (ESD) in both long and short axes and ventricular wall thickness from these values, we also calculated fractional shortening as a measure of contractile function. We found that sorafenib and sunitinib but not gefitinib (all at 0.5 μmol/L) significantly reduced ventricular wall thickness ($P<0.01$ for sorafenib and $P<0.001$ for sunitinib versus vehicle or gefitinib) (Table 1). Contractile function, as expressed by fractional shortening, was markedly reduced by both drugs ($P<0.0001$ for sorafenib and sunitinib versus vehicle or gefitinib) (Table 1). Cardiac dilatation, as determined by EDD, was also pronounced in sunitinib-treated fish ($P<0.01$ versus vehicle or gefitinib; Table 1). In summary, both sorafenib and sunitinib but not gefitinib led to reduced cardiac wall thickness, ventricular dilatation, and markedly impaired contractile function.

Sorafenib Induces Cell Death in NRVMs and Fish In Vivo

We then explored possible mechanisms underlying the contractile dysfunction. Mechanisms of sunitinib-induced cardiotoxicity have been the subject of prior reports and therefore those studies were not repeated here. To elucidate the mechanisms underlying the cardiotoxicity of sorafenib, we used isolated NRVMs. We found that sorafenib dose-dependently induced cell death in NRVMs as determined by 2 different approaches, TUNEL staining and the ToxiLight assay (Figure 3).

We then asked whether the death of NRVMs translated to findings in the fish in vivo by asking whether the thinning of the ventricular walls after sorafenib treatment might be due in part to cardiomyocyte loss. To address this question, we used Cmlc2:dsRed-nuc transgenic fish in which the cardiomyocyte nucleus is red, allowing quantification of total cardiomyocyte number. We found a highly significant reduction in number of ventricular myocytes in the sorafenib-treated fish at all doses (Figure 4A).

We next asked, based on the findings in NRVMs exposed to sorafenib, if this reduction in cardiomyocyte number might be due in part to apoptosis. We used acridine orange (AO) staining, a widely used method to detect apoptosis in zebrafish. We treated fish with 0.5 μmol/L drug at 2 dpf and then stained whole fish at 3 dpf with the vital dye acridine orange (AO). AO positive cells were observed in the myocardium of the fish. Representative images of AO-positive cell nuclei are shown in Figure 4B.
been implicated in survival signaling in the heart21 (though That said, Raf-1 (and B-Raf) are targets of sorafenib and have it difficult to pinpoint the specific target(s) mediating toxicity. This makes selectivity across a panel of 317 kinases, sorafenib binds to cardiotoxicity. In a quantitative analysis of kinase inhibitor We next examined signaling pathways regulating sorafenib pathway mediating

to a persistent decrease in the basal level of pERK and this was dose-dependent, consistent with inhibition of Raf by sorafenib. Furthermore, pretreatment with sorafenib blocked ERK phosphorylation induced by growth factors such as insulin-like growth factor-1 and insulin, as well as oxidative stress induced by hydrogen peroxide (H$_2$O$_2$) (Figure 5C). Surprisingly, sorafenib failed to block activation of ERK by phenylephrine (PE), identifying a novel Raf-independent pathway mediating adrenergic agonist-induced ERK activation that will be addressed further below. We next asked whether sorafenib-induced cardiomyocyte apoptosis is at least partially mediated by inhibition of ERK. We used 3 distinct and selective inhibitors of MEK1/2, kinases that are immediately downstream of Raf-1/B-Raf and are responsible for the phosphorylation and activation of ERK1/2. Twenty-four-hour treatment of NRVMs with MEK1/2 inhibitors (PD184352, UO126, or PD98059) decreased the level of pERK and induced apoptosis by 2- to 3-fold (Figure 6A). These data confirm that three distinct (and selective) MEK1/2 inhibitors could recapitulate the proapoptotic effect of sorafenib on cardiomyocytes, suggesting that sorafenib-induced cell death is mediated by an on-target effect: inhibition of the Raf/MEK/ERK pathway.

Molecular Mechanisms of Sorafenib-Induced Cell Death
We next examined signaling pathways regulating sorafenib cardiotoxicity. In a quantitative analysis of kinase inhibitor selectivity across a panel of 317 kinases, sorafenib binds to more than 15 kinases with nanomolar potency. This makes it difficult to pinpoint the specific target(s) mediating toxicity. That said, Raf-1 (and B-Raf) are targets of sorafenib and have been implicated in survival signaling in the heart (though controversies remain22). Therefore, we asked whether sorafenib-induced cardiotoxicity is mediated, at least in part, by the inhibition of Raf/ERK signaling. We first confirmed that sorafenib inhibited ERK activation in zebrafish (Figure 5A). We then confirmed that sorafenib inhibited ERK activation in NRVMs (Figure 5B). Treatment with sorafenib led to a persistent decrease in the basal level of pERK and this was dose-dependent, consistent with inhibition of Raf by sorafenib. Furthermore, pretreatment with sorafenib blocked ERK phosphorylation induced by growth factors such as insulin-like growth factor-1 and insulin, as well as oxidative stress induced by hydrogen peroxide (H$_2$O$_2$) (Figure 5C). Surprisingly, sorafenib failed to block activation of ERK by phenylephrine (PE), identifying a novel Raf-independent pathway mediating adrenergic agonist-induced ERK activation that will be addressed further below. We next asked whether sorafenib-induced cardiomyocyte apoptosis is at least partially mediated by inhibition of ERK. We used 3 distinct and selective inhibitors of MEK1/2, kinases that are immediately downstream of Raf-1/B-Raf and are responsible for the phosphorylation and activation of ERK1/2. Twenty-four-hour treatment of NRVMs with MEK1/2 inhibitors (PD184352, UO126, or PD98059) decreased the level of pERK and induced apoptosis by 2- to 3-fold (Figure 6A). These data confirm that three distinct (and selective) MEK1/2 inhibitors could recapitulate the proapoptotic effect of sorafenib on cardiomyocytes, suggesting that sorafenib-induced cell death is mediated by an on-target effect: inhibition of the Raf/MEK/ERK pathway.

| Table 1. Videomicroscopic Measurements in Fish 5 dpf That Were Treated at 2 dpf With Vehicle or 0.5 μmol/L KIs |
|---|---|---|---|---|---|---|
| | Long Axis | | | Short Axis | |
| | n | EDD | ESD | FS=(EDD–ESD)/EDD | Wall Thickness | EDD | ESD | FS=(EDD–ESD)/EDD |
| Vehicle | 6 | 98.7 | 40.7 | 0.59 | 40.4 | 62.4 | 24.0 | 0.61 |
| Gefitinib | 6 | 98.0 | 39.2 | 0.59 | 39.9 | 55.5 | 22.4 | 0.59 |
| Sorafenib | 12 | 102.9 | 69.7 | 0.33 | 27.1 | 61.3 | 37.7 | 0.39 |
| Sunitinib | 12 | 117.1 | 78.6 | 0.33 | 23.9 | 60.8 | 42.6 | 0.30 |

Ventricular wall thickness along the long axis, end-diastolic dimension (EDD), and end-systolic dimension (ESD) in both long and short axes were quantified and expressed as arbitrary units. Fractional shortening (FS) was then calculated as a measure of contractile function. For all video images, 100 arbitrary units equal 0.268 mm.

*P<0.01 versus vehicle or gefitinib.
†P<0.05 versus sorafenib.

ventricles are shown in Figure 4B. With sorafenib treatment, 38.9% of fish demonstrated AO-positive myocardium, and this was significantly higher than the percent of AO-positive hearts treated with vehicle (23.1%) or gefitinib (26.1%) (P<0.01 and 0.05, respectively; Figure 4B, inset graph). Of note, the percent of fish with AO-positive hearts treated with sunitinib (32.2%) reached statistical significance when compared with vehicle (P=0.046) but not when compared with gefitinib (P=0.14; Figure 4B, inset graph). These data suggest that apoptosis plays a role in sorafenib (and sunitinib)-mediated cardiotoxicity.

**Figure 3.** Sorafenib dose-dependently induces cell death in NRVMs. Cells were plated in 8-well chamber slides and treated with vehicle or 0.5–5 μmol/L sorafenib for 24 hours. Apoptosis was assessed with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. Cell death was also assessed based on the release of adenylate kinase from damaged cells into culture supernatants (ToxiLight). *P<0.05 versus vehicle; n=3 independent experiments.
To explore this further, we used an adenovirus that expresses a constitutively active form of MEK1/2 (MEK-DD) to attempt to rescue sorafenib cardiotoxicity. We found that transduction of NRVMs at 10 to 40 MOI of MEK-DD adenovirus increased pERK by 3- to 7-fold (Figure 6B) and markedly reduced sorafenib-induced apoptosis (Figure 6C). These data suggest that inhibition of ERK contributes significantly to sorafenib-induced cardiomyocyte apoptosis in mammalian cardiomyocytes just as it appears to do in zebrafish and probably is responsible for the increased apoptosis that we observed in the myocardium of sorafenib-treated fish.

Finally we examined the role of the novel Raf-independent signaling pathway that activates ERK downstream of α-adrenergic agonists (Figure 5C). We first found that although this pathway bypassed Raf to activate ERK, activation of ERK remained entirely dependent on MEK1/2 because the MEK inhibitors abrogated PE-induced ERK activation (Figure 6D). More importantly, we found that treatment of cardiomyocytes with the α-adrenergic agonist, PE, markedly reduced sorafenib-induced cell death (Figure 6E). These data suggest that α-adrenergic signaling may effectively protect against sorafenib-induced cardiotoxicity. To evaluate the importance of this pathway in vivo, we used a loss-of-function approach in the fish by treating them with the α-adrenergic receptor antagonist, prazosin. Prazosin alone had no effect on ventricular function, but, when given together with sorafenib, prazosin significantly exacerbated sorafenib-induced cardiac dysfunction (Table 2).

Discussion

We introduce zebrafish as a model to examine cardiotoxicity, or lack thereof, of 3 FDA-approved kinase inhibitors that are being used to treat patients with various malignancies.23 We believe this is the first use of zebrafish for this purpose, and,
based on our findings, suggest that the use of this model organism should be further evaluated for preclinical testing. We also identify the Raf/MEK/ERK pathway as a key target of sorafenib and demonstrate that inhibition of this pathway probably mediates, at least in part, the cardiotoxicity associated with this agent. Finally, we identify a novel Raf-independent pathway downstream of α-adrenergic receptors that leads to ERK activation and, in isolated cardiomyocytes and fish in vivo, protection against sorafenib cardiotoxicity.

It seems clear that preclinical models of kinase inhibitor-induced cardiotoxicity are inadequate because a number of kinase inhibitors have been withdrawn in the late stages of development, at great expense to the companies developing them. Attempts to utilize various cell lines and primary cells...
have been helpful, but the consensus appears to be that an in vivo system is needed for optimal detection. That said, the problems with rodent models have been mentioned above. Although rodent models have been used successfully in studies with anthracyclines, results with KIs have been less consistent. For example, we were unable to demonstrate LV dysfunction in mice treated with sunitinib even when we added an additional pressure stress on the heart. Similar findings have been reported in rats with sunitinib.27

Because of these issues, we examined whether zebrafish might be a viable model. Zebrafish have been used extensively in embryology and genetics, establishing a well-conserved linkage to mammalian genetics. They have also been used to predict drugs that may cause teratogenic effects and conduction defects including QT interval prolongation. Although the zebrafish heart has only 2 chambers, zebrafish cardiomyocytes, as in mammals, express voltage-gated sodium channels, L-type and T-type calcium channels, and potassium channels.

With the zebrafish system, we were able to differentiate an agent with clear evidence of cardiotoxicity in the clinic (sunitinib) from one with no signal for cardiotoxicity (gefitinib). The system also predicts cardiotoxicity of sorafenib. It should be noted that the increased death and abnormal body habitus in zebrafish treated with sorafenib only occurred at high concentrations of drug, and, because these fish had multiple abnormalities, it is impossible to differentiate cardiac from noncardiac mortality. At lower concentrations, neither death nor abnormal body habitus was seen, but cardiotoxicity was obvious. To our knowledge, no prospective studies of sorafenib cardiotoxicity that have carefully examined LV function have been performed. Our findings, particularly given the relatively low concentrations of sorafenib we used, suggest that postmarketing surveillance for sorafenib cardiotoxicity through registries might be prudent, especially as use of the agent broadens into cancer patients with cardiovascular comorbidities.

Although our findings might suggest a gradient of cardiotoxicity between sorafenib and sunitinib (more cardiac dilation and reduced fractional shortening with sunitinib; Table 1), these comparisons may not be valid given differences in pharmacokinetics and pharmacodynamics between the drugs.
Table 2. Videomicroscopic Measurements in Fish 5 dpf That Were Treated at 2 dpf With Vehicle, 0.5 μmol/L Sorafenib Only, or 0.5 μmol/L Sorafenib Supplemented With Prazocin

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<th>Long Axis</th>
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<td></td>
<td>n</td>
<td>EDD</td>
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<tr>
<td>Vehicle</td>
<td>9</td>
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<tr>
<td>±5.8 ±1.3 ±0.2 ±0.3</td>
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<tr>
<td>Sorafenib only</td>
<td>12</td>
<td>101.7</td>
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<tr>
<td>±4.8 ±4.6* ±0.03* ±0.4*</td>
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<tr>
<td>Sorafenib</td>
<td>10</td>
<td>96.4</td>
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<tr>
<td>+5 μmol/L PZ</td>
<td>±6.1</td>
<td>±4.2*</td>
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<tr>
<td>+25 μmol/L PZ</td>
<td>11</td>
<td>97.6</td>
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<tr>
<td>±4.1 ±3.2* ±0.02*†</td>
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<tr>
<td>25 μmol/L PZ only</td>
<td>10</td>
<td>89.0</td>
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<tr>
<td>±3.2 ±3.5 ±0.03 ±0.7</td>
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EDD indicates end-diastolic dimension; ESD, end-systolic dimension; FS, fractional shortening; and PZ, prazocin.

*P<0.01 versus vehicle.
†P<0.05 versus sorafenib.

Until concentrations of the agents in the target tissues of interest in patients and model organisms are known, one cannot reliably compare cardiotoxicity of two agents in the model organism. One can only say whether cardiotoxicity is present or not.

One additional caveat to the use of zebrafish (or any model organism) for predicting cardiotoxicity include the possibility of amino acid sequence differences between fish and human at the ATP pocket of targeted kinases, where most KIs interact. In this scenario, cardiotoxicity could be either underestimated or overestimated, based on the avidity of binding of the compound to the pocket. This will obviously necessitate careful sequence comparisons between zebrafish and human genomes, and projections as to how specific sequence differences may impact kinase inhibitor binding.

To further develop the zebrafish model for cardiotoxicity testing of KIs, we believe that the next step will be to validate this model with other approved agents for which cardiotoxicity profiles are known. The next step after that could be to prospectively examine new agents in early phase clinical trials and then follow the patients in those trials for signals of cardiotoxicity. Thus, more likely, we will be left with examining approved agents, because it is very rare to gain access to agents while they are in development.

Finally, we identified a protective effect of α-adrenergic signaling against sorafenib-induced cardiotoxicity that is mediated by a novel signaling pathway, bypassing Raf-1/B-Raf but acting through MEK1/2, to activate ERKs. This pathway appears to be uniquely activated by α-adrenergic stimuli because Raf-1/B-Raf was necessary for activation of ERKs downstream of growth factor receptors and oxidative stress. Our findings of a protective role of α-adrenergic signaling against toxicity of sorafenib in cardiomyocytes are consistent with earlier studies demonstrating a protective role of α-adrenergic signaling in the heart exposed to severe pressure stress induced by banding of the aorta in the mouse.28

Collectively, our data raise potential concerns about the simultaneous use of α-adrenergic antagonists such as prazosin (used in patients with benign prostatic hypertrophy) and sorafenib. Given the common usage of α-adrenergic antagonists in clinical practice (~50% of men over 60 years of age have benign prostatic hypertrophy and many require treatment),29 the potential consequences of the concomitant use of these agents with sorafenib should be considered.

Acknowledgments

We acknowledge the support of the zebrafish facility of Thomas Jefferson University.

Sources of Funding

This work was supported by National Heart, Lung, and Blood Institute grants HL061688 (to T.F.) and HL091799 (to W.J.K. and T.F.).

Disclosures

None.

References

Novelty and Significance

What Is Known?
- Small-molecule kinase inhibitors (KIs) have demonstrated success in treating cancers; however, some of them are cardiotoxic.
- The current preclinical models of Ki-induced cardiotoxicity, including rodents, are inadequate.
- The zebrafish has been adopted as a vertebrate model for human cardiovascular disease research and drug discovery, the latter mostly focused on QT prolongation.

What New Information Does This Article Contribute?
- Zebrafish may be a valuable model to predict possible Ki-induced cardiotoxicity before human use.
- Inhibition of Raf/MEK/ERK pathway is a key mechanism of sorafenib-induced cardiotoxicity.
- A novel Raf-independent pathway from α-adrenergic receptors to ERK activation appears to protect against sorafenib-induced cardiotoxicity.

With the explosion in development of KIs targeting kinases that are mutated or overexpressed in cancer, cardiotoxicity with KIs will continue to plague drug development until reliable preclinical screening strategies are developed. Unfortunately, current preclinical models of Ki-induced cardiotoxicity are inadequate. We introduce zebrafish as a model for this purpose. We found that the zebrafish can discriminate a Ki with little or no cardiotoxicity (gefitinib) from one with demonstrated cardiotoxicity (sunitinib). Sorafenib, like sunitinib, leads to cardiomyocyte degeneration, whereas zebrafish can discriminate a KI with little or no cardiotoxicity. Our findings suggest that the use of this model organism should be further evaluated for preclinical testing. Collectively, our data raise concerns about the simultaneous use of α-adrenergic antagonists and sorafenib in clinical practice.
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Circ Res. published online October 13, 2011;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Methods:

Materials
Pharmacological agents PD98059 (Sigma), PD184352 (Santa Cruz Biotechnology), UO126, sorafenib, gefitinib and sunitinib (LC Laboratories, Woburn, MA) were dissolved in DMSO. IGF-1 (BD Biosciences) and insulin (Sigma) were dissolved in phosphate buffered saline (PBS) and in 0.01 M hydrochloric acid (HCl), respectively. Phenylephrine chloride (PE), prazosin hydrochloride and hydrogen peroxide (H₂O₂) (Sigma) were dissolved in ddH₂O.

Zebrafish embryo harvesting and maintenance
Zebrafish use and handling at the Thomas Jefferson University (TJU) Zebrafish Facility was approved by the Institutional Animal Care and Use Committee at TJU. Wild-type adult fish were mated in embryo collection tanks. A transgenic line (TG:VEGFR2-GRCFP, kindly provided by Amy Rubinstein at Zygogen LLC, Atlanta, GA) was employed to assess vascular morphology of zebrafish, in which expression of a green reef coral fluorescent protein (G-RCFP14) is driven by the promoter of vascular endothelial growth factor receptor 2 (VEGFR2) gene. Another transgenic line (TG: Cmlc2::dsRed-nuc, kindly provided by Calum McRae of Massachusetts General Hospital) was employed to quantify cardiomyocytes, in which expression of a Discosoma red fluorescent protein (Ds-Red) in nuclei is driven by the promoter of the cardiac myosin light chain 2 (Cmlc2) gene.

Viable embryos were washed with embryo medium (EM) and sorted (30 embryos per 60-mm dish in 10ml EM) at the one- to two-cell developmental stage (approximately 0.5-1 h post fertilization [hpf]), and then were maintained under normoxic conditions at 28.5°C to enable normal development. EM was changed after dechorionation at 24-48hpf and again at 72-96 hpf.

Neonatal rat ventricular cardiomyocytes
Neonatal rat ventricular myocytes (NRVMs) were isolated from 2- to 3-day old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and cultured as previously described. The isolated myocytes were plated onto Primaria cell culture plates (Becton and Dickinson) or Lab-Tek™ Chamber Slides (Nunc) pre-coated with laminin.

NRVMs were treated with the various agents for the times, and at the concentrations, noted in the figures and legends. For all the experiments with sorafenib treatment involving NRVMs, cells were placed in DMEM media that were supplemented with 1-2% fetal bovine serum (FBS). For all studies examining ERK activation, NRVMs were starved in serum-free medium overnight, and then were pre-treated with KIs for 50 min followed by stimulation with IGF-1 (50 ng/ml), insulin (5 ug/ml), H₂O₂ (50 μM) or phenylephrine (10 μM) for 20 min.

Analysis of treatment effects on zebrafish survival and gross morphology
Zebrafish were treated with the various KIs at the concentrations and for the times noted in the figure legends. Unless otherwise noted, treatment occurred at 2dpf. Toxicity analyses were conducted by monitoring survival and morphology of zebrafish for up to 7 dpf. At the time of examination, zebrafish were anesthetized with a 1:100 dilution of 4 mg/mL tricaine methanesulfonate (Sigma), and then morphology was assessed visually using a light transmission microscope (Olympus BX51, Germany) at 12.5× magnification. Images were recorded using a Zeiss AxioCam camera and AxioVision 3.0 software. Survival of zebrafish was assessed visually by light microscopy. The criterion for fish survival was the presence of cardiac contractions. Representative images of transgenic fish for assessment of angiogenesis were also captured.
Heart videos and quantification

For zebrafish that would be examined by videomicroscopy, embryo medium containing 1-phenyl-2-thiourea (PTU, 50 μM) was used to suppress pigmentation in developing embryos. Diluted DMSO or 0.5 uM drug solutions were added to 2dpf fish, medium was changed at 4dpf without further drug treatment and videos were taken at 5dpf. For treatments with prazosin, 0.5 uM sorafenib solutions supplemented with various doses of prazosin (dissolved in dH2O) were added to 2dpf fish. Due to the short half-life of prazosin (2~3 hours), prazosin was replenished twice every day on day 3 and day 4. 5dpf fish were immobilized by placing them on 3% methylcellulose on a glass depression slide. After they were adjusted to an “abdomen up” position, video files were recorded on a Sensi Cam with Streampix 3 software, at a frame rate of 20fps (frame per second) over 5 seconds. Quantification of ventricular wall thickness (along long axis), end-diastolic dimension (EDD) and end-systolic dimension (ESD) in both long and short axis were done with Image J (NIH).

Cell death assays

To assay apoptosis in zebrafish, 2dpf fish were treated with drugs for 24 hrs. Then the live whole fish were stained for 20 min at room temperature using 1μg/mL of acridine orange dye (Sigma) and rinsed five times with embryo medium as described previously 4. Zebrafish images were taken under a Leica MZ16FA microscope (Leica, Wetzlar, Germany) with GFP filter using a Leica DFC300FX camera (Leica, Wetzlar, Germany). The images were processed using Leica Application Suite V3.3.0 (Leica, Wetzlar, Germany) and further converted to grayscale images using Adobe Photoshop CS2.

For cell death in NRVMs, we employed two approaches. Staining of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; kit from Millipore) was performed following the manufacturer’s instructions. Apoptotic cells were visualized with a Nikon Eclipse 90i microscope and software from NIS-elements was used to record images. We also utilized the ToxiLight assay (Lonza) that quantifies loss of sarcolemmal integrity. Loss of integrity leads to the release of adenylate kinase into the media and this is measured in culture supernatants employing a bioluminescense assay 3.

Cardiomyocyte number

Cmlc2::dsRed-nuc zebrafish were raised in embryo medium containing PTU, anesthetized, mounted in Lebovitz’s L15 medium-10% fetal bovine serum (FBS) on a glass slide, and held in place with a coverslip. Images of the hearts were taken with a QIMAGING camera mounted on an inverted microscope and iVision software, and the number of red fluorescent nuclei was counted with NIS-elements AR 2.30 (NIKON Instruments).

Immunoblotting and densitometry

To make lysates from NRVMs, RIPA buffer supplemented with additional Phosphatase and protease inhibitors was used. Protein concentration were normalized and loaded on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell). Next, the membranes were blocked with blocking buffer (Li-COR, Lincoln, Nebraska, USA) for 30min and incubated at 4°C overnight with primary antibodies as indicated. The following day, membranes were washed three times and incubated with appropriate Alexa Fluor 680 dye-labeled secondary antibodies (Invitrogen) for 1 hour at room temperature. Antibody binding was detected using an Odyssey Infrared Imaging System (LI-COR). Protein levels were quantified with Image J (NIH). The signals were normalized to that of either GAPDH or total ERK to correct for potential differences in protein loading.

To make lysates from 3dpf zebrafish (~30 fish per treatment), fish were collected into 1.5ml eppendorf tubes. Embryo medium was completely removed followed by washing with
70% PBS, and then the same lysis buffer used for cells was added to each tube of fish followed by sonication. Fish lysates were loaded onto SDS-PAGE. Procedures that followed were identical to those described above for immunoblotting lysates from NRVMs.

Antibodies employed were as follows: rabbit anti-ERK1/2 pan antibody was from Invitrogen (Camarillo, CA); phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mouse or rabbit antibodies and MEK1/2 antibody were from Cell Signaling (Beverly, MA, USA); GAPDH antibody was from Fitzgerald Industries International (Concord, MA, USA).

Adenoviral gene transfer
NRVMs were transduced with an adenovirus encoding a constitutively-active form of MEK1 (MEK1-DD, kindly provided by Alessandro Alessandrini of Massachusetts General Hospital \(^5, 6\)) or with an adenovirus encoding green fluorescent protein as a control, at a multiplicity of infection of 10-40 MOI for 24 hours prior to performing experiments.

Statistical analysis
Differences between data groups were evaluated for significance using Student’s t-test of unpaired data or one-way ANOVA followed by Tukey’s post-test (significance level set at P<0.05). Categorical data were analyzed using Fisher’s exact test. All experiments were repeated at least three times and the data are presented as mean ± SEM unless noted otherwise.

References:
Supplemental Figures and Legends:

Supplemental Figure I: Representative images of wild-type fish at 5dpf that had been treated with vehicle, 5uM sorafenib, sunitinib, or gefitinib at 2dpf. Very noticeable body malformations and pericardial edema were observed in fish treated with sorafenib or sunitinib (but not gefitinib).

Supplemental Figure II: Representative images of transgenic (TG:VEGFR2-GRCFP) fish at 5dpf that had been treated with vehicle, 0.5uM sorafenib, sunitinib, or gefitinib at 2dpf. No abnormalities of the vasculature were observed in any of the KI-treated fish.