Downregulation of Stanniocalcin 1 is Responsible for Sorafenib-Induced Cardiotoxicity

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ABSTRACT

Sorafenib is associated with adverse cardiac effects, including left ventricular dysfunction. However, the precise mechanism remains unclear. Here, we aimed to establish the genes responsible for this cardiotoxicity using zebrafish and human cardiomyocytes. Fluorescent cardiac imaging using pigmentless zebrafish with green fluorescent protein hearts revealed that the ventricular dimensions of the longitudinal axis with sorafenib were significantly shorter than those of the control group. Transcriptome analysis of their hearts revealed that stanniocalcin 1 (stc1) was downregulated by sorafenib. stc1 knockdown in zebrafish revealed that reduction of stc1 decreased the longitudinal dimensions of zebrafish ventricles, similar to that which occurs during sorafenib treatment. STC1 downregulation and cytotoxicity were also seen in human cardiomyocytes exposed to sorafenib. To clarify the molecular function of stc1 in sorafenib-induced cardiotoxicity, we focused on oxidative stress in cardiomyocytes treated with sorafenib. Reactive oxygen species (ROS) production significantly increased in both species of human cardiomyocytes and zebrafish exposed to sorafenib and STC1 knockdown compared with the controls. Finally, we found that forced expression of stc1 normalized impairment, decreasing the longitudinal dimensions in zebrafish treated with sorafenib. Our study demonstrated that STC1 plays a protective role against ventricular dysfunction and ROS overproduction, which are induced by sorafenib treatment. We discovered for the first time that STC1 downregulation is responsible for sorafenib-induced cardiotoxicity through activated ROS generation.

Key words: sorafenib; stanniocalcin 1; cardiotoxicity; zebrafish; reactive oxygen species

Cardiotoxicity is one of the most significant adverse effects of cancer treatment, and is responsible for considerable morbidity and mortality (Adão et al., 2013). Cardiac function is maintained by an intricate cycle of molecular signaling events that significantly overlap the signaling pathways of tumor growth. For example, many protein kinases are constitutively active in cancer cells, through mutations, overexpression, or translocation (Force et al., 2007). However, the use of certain chemotherapeutics for the treatment of cancer is associated with a risk of adverse effects on cardiac function (Force et al., 2007).
These kinase inhibitors can be cardiotoxic in a subset of patients (Force and Kolaja, 2011). With consideration to the therapeutic efficacy of these small molecular inhibitors, it is crucial to clarify the precise mechanism of drug-induced cardiotoxicity. Sorafenib is the first oral multikinase inhibitor that can inhibit v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), fms-like tyrosine kinase (FLT)4, also known as vascular endothelial growth factor receptor (VEGFR)3, FLT3, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), and platelet-derived growth factor receptor (PDGFR). The safety and efficacy of sorafenib in patients with advanced hepatocellular carcinoma (HCC) was demonstrated in two phase III randomized, double-blind, placebo-controlled trials, the Sorafenib HCC Assessment Randomized Protocol (SHARP) and Asia-Pacific (AP) trials (Cheng et al., 2009; Llovet et al., 2008b), thereby establishing sorafenib as the standard systemic therapy for advanced HCC (Bruix et al., 2011; Llovet et al., 2008a; Miyahara et al., 2014). The 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 study that examined baseline and serial LVEF determinations in patients on sorafenib reported that mean LVEF declined by only 0.8–1.2 EF percent (Cheng et al., 2011; Tolcher et al., 2011). These authors reported that the effects on LVEF were modest and were unlikely to be of clinical significance, but 13% of patients had significant declines in ejection fraction (EF) (>10 EF points). In the study by Schmidinger et al. (2008), the incidence of reduced LVEF for sorafenib was reported as 5%. Several mechanisms of sorafenib-induced cardiotoxicity have been reported, including inhibition of the RAF/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (Cheng et al., 2011; Mello et al., 2011), and disruption of VEGF-VEGFR signaling through inhibition of circulating VEGF and inhibition of PDGFRs (Hasinoff and Patel, 2010). Recently, Cheng et al. reported that sorafenib-induced cardiotoxicity may be preventable by activation of α-adrenergic signaling. Phenylephrine can activate ERK independently of RAF (Cheng et al., 2011). Further studies are required to give more options for the prevention of sorafenib-induced cardiotoxicity.

Zebrafish have emerged as a useful animal model to clarify the molecular mechanisms of various cardiac diseases, including drug-induced cardiotoxicity (Lal et al., 2013). Zebrafish in which a certain gene product associated with human heart failure was reduced, showed a decrease in EF, similar to the human phenotype (Seguchi et al., 2007). We have also developed a fluorescent cardiac imaging of pigmentless zebrafish heart that can assess the cardiac function in various physiological and pathophysiological situations (Umemoto et al., 2013). In this study, we applied functional cardiac imaging and transcriptome analysis to zebrafish to clarify the molecular mechanism of sorafenib-induced cardiotoxicity. These analyses, combined with the validation studies using human cardiomyocytes, revealed that downregulation of STC1, a gene that has cardioprotective function (Koizumi et al., 2007; Liu et al., 2012), is responsible for sorafenib-induced cardiotoxicity.

**MATERIALS AND METHODS**

This study was approved by the Ethics Committee of Mie University School of Medicine, Japan, and involved the use of zebrafish embryos as experimental animals. Animal care was also in accordance with the NIH guidelines. Experimental animals. We obtained a pigmentless zebrafish line nacre (Lister et al., 1999) from Zebrafish International Resource Center (ZIRC; Eugene, OR) and a transgenic zebrafish line SAG4A (Kawakami et al., 2004) expressing enhanced green fluorescent protein (EGFP) in the heart from the National Institute of Genetics (Mishima, Japan). We crossed nacre and SAG4A to create MieKomachi 009 (MK009) (Supplementary Figure S1A). MK009 were bred and maintained as described by Westerfield (2007). Zebrafish were raised at 28.5 ± 0.5°C with a 14/10 h light/dark cycle. Embryos were obtained via natural mating and cultured in egg water (1.5 ml stock salts added to 11 distilled water).

Sorafenib exposure. We filled a 6-well plate (Falcon, New York, NY) with 2 ml egg water per well (10 fish of 3 days post fertilization [dpf] in each of 6 baskets). Fifty zebrafish per group were analyzed. We administered sorafenib (1 or 3 μM) or 1% dimethyl sulfoxide (DMSO) to MK009 from 3 to 10 dpf, and changed the medium every day. MK009 were immobilized by placing them on 3% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA) on a glass slide (Matsunami, Tokyo, Japan). Images were captured using a biological microscope (MZFLA; Leica, Wetzlar, Germany) with a digital camera (DP2; Olympus, Tokyo, Japan). The criterion for survival was the presence of cardiac contractions.

Cardiac imaging analysis. We administered 1 μM sorafenib or 1% DMSO to MK009 from 72 to 96 or 120 h post-fertilization (hpf). MK009 were immobilized by placing them on 3% methylcellulose in a microscopy chamber (μ-Slide 8 well uncoated; ibidi, Munich, Germany) without anesthesia. After they were adjusted to a “head left” position, movie files were recorded on Image Xpress MICRO (Molecular Devices, Sunnyvale, CA, USA), at a frame rate of 33.5 frames/second for ~6s.

We used MacBiophotonics Image J (NIH, Bethesda, MD) (Collins, 2007) to process the original records for the measurement of cardiac performance using GFP heart imaging, as described by Umemoto et al. (2013). To make M-mode images in the longitudinal (long) or short axes of the ventricle, the intersection of horizontal and vertical lines was positioned at the center of mass according to our preliminary experiment (Umemoto et al., 2013). The ventricular end-systolic dimension (VDs) and the ventricular end-diastolic dimension (VDd) were measured from the M-mode images (Supplementary Figure S1B). VDs and VDd were obtained at maximal inward and outward excursions, respectively, of the ventricular wall in the M-mode images. We measured VDs and VDd along the short axis (short VDs and short VDd) and the longitudinal axis (long VDs and long VDd). For each determination, 5 diastoles and systoles were analyzed. The end-diastolic volume (EDV) and end-systolic volume (ESV) were calculated using the formula for a prolate spheroid (4πa×b³/3) (Jacob et al., 2002). The stroke volume (SV) was obtained by subtracting the ESV from the EDV. The EF was calculated as SV/EDV and the percentage fractional shortening (FS) was calculated from the formula: 100 (short VDd – short VD)/short VD.
Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), and quantified using a spectrophotometer (Nano Drop ND-100; Wilmington, DE). In total, 700 of total RNA was converted into labeled cRNA using the Low Input Quick Amp Labeling Kit (Agilent Technologies). Cy3-labeled cRNA (1.65 μg) was hybridized to Agilent Zebrafish Whole Genome Oligo Microarrays (G2519F) according to the manufacturer’s protocol. The hybridized microarrays were scanned by DNA Microarray Scanner (G2565BA; Agilent Technologies) and analyzed using Feature Extraction software (Agilent Technologies). The data were normalized using Ag6kk4PreProcess. RankProd analysis was performed using Bioconductor to identify differentially expressed genes between two groups by calculating the false-discovery rate (FDR). Differentially expressed genes (FDR <10%) were then converted to human orthologs using the Life Science Knowledge Bank (World Fusion, Tokyo, Japan). These genes were subjected to Pathway Studio, which is a program used to visualize and analyze biological pathways and gene regulation networks. This program includes the ResNet database of >500,000 functional relationships between genes and functional entities such as heart failure. The microarray dataset has been deposited in GEO as GSE61155.

Cell preparations. Human adult primary cardiomyocytes were purchased (PromoCell, Heidelberg, Germany) and cultured according to the manufacturer’s instructions. Cardiomyocytes were grown for 2 weeks in a medium consisting of Myocyte Growth Medium Kit (PromoCell) supplemented with 5% fetal calf serum, epidermal growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml), and insulin (5 μg/ml) at 37°C in a 5% CO2 humidified incubator, and the medium was changed every day.

siRNA transfection. Stealth siRNAs ( Stealth RNAi Negative Control Med GC [12935-300] and Stealth RNAi for STC1 (STC1siRNA1:HSS110297 and STC1siRNA2:HSS186139) were purchased from Life Technologies, and were denoted as NCsiRNA and STC1siRNA-2, respectively. Three biological replicates were prepared for each group. The sequences of siRNAs used in this study are shown in Supplementary Table S1. Cultured cells were transfected with siRNAs (50 nM final concentration) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s protocol.

Quantitative PCR Analysis. Total RNA from heart tissues and whole body of zebrafish was purified as described above. Total RNA from human cardiomyocytes was also purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). First-standard cDNA was prepared with 200 ng total RNA and used to generate cDNA using an iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative polymerase chain reaction (qPCR) was performed using an ABI Prism 7300 (Life Technologies) with SYBR Green Real-time PCR Master Mix Plus (Toyobo, Osaka, Japan). The thermal cycling conditions comprised an initial step at 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 45 s. The sequences of primers used in this study are shown in Supplementary Table S1. Gene expression levels were calculated using the standard curve method.

Injections of morpholino and/or stc1 mRNA. The stc1 antisense morpholino oligonucleotides (MOs) and standard control MO were purchased from Gene Tools (Philomath, OR). The stc1 antisense MO was designed to splice out the second exon of stc1 (zgc:153307). The sequences of MOs used in this study are shown in Supplementary Table S1.

The zebrafish stc1 (amino acids 1–250) obtained from ZGC clone 153307 (Source BioScience, Nottingham, UK) was subcloned into the EcoRI and XbaI sites of the plasmid pCS2+ (Addgene, Cambridge, MA). The sequence of the construct was confirmed by DNA sequencing (FASMAC, Kanagawa, Japan). The sequence containing full-length cDNA for stc1 was amplified by PCR using the primer pairs: SP6 and T3. The PCR product was used as the template for RNA synthesis. The stc1 mRNA was synthesized using the mMessage mMachine transcription kit for SP6 RNA polymerase (Life Technologies).

For knockdown of stc1, a solution containing 0.3 mM stc1 MO was injected into 1- to 4-cell stage embryos. A solution containing 0.3 mM control MO was also injected into zebrafish as a control. For the rescue experiment, a solution containing mRNA (0.87 μM) with or without 0.3 mM stc1 MO was injected into 1- to 4-cell stage embryos. These embryos were raised in egg water at 28.5°C until 96 hpf.

Reactive oxygen species measurement. In zebrafish experiments, we administered 1 μM sorafenib or 1% DMSO to MK009 from 72 to 74 hpf. DHE (Life Technologies) was dissolved in DMSO and used at a final concentration of 10 μM in egg water in the presence of 0.1% DMSO. Zebrafish were incubated with DHE for 15 min in the dark chamber (Ogrunc et al., 2014), rinsed in egg water twice, and anesthetized using 2-phenoxy-ethanol, then immediately imaged under a Nikon stereomicroscope SMZ 25 (Tokyo, Japan) equipped with filters for DS-Red. Zebrafish fluorescent intensity of zebrafish whole body was quantified using the MacBiophotonics Image J (NIH).

Following 0.3 μM sorafenib or vehicle administration for 1 h, cultured cardiomyocytes were incubated with cell-permeable redox-sensitive fluorophore CellRox Deep Red detection reagent (Life Technologies) in a microscopy chamber (u-Slide 8 well uncoated; ibidi, Munich, Germany), at a concentration of 5 μM for 30 min. Hoechst 33342 dye (10 μM; Dojin, Kumamoto, Japan) was used to visualize the nuclei. Cardiomyocytes were washed with warm PBS twice. Cell images were acquired using ImageXpress MICRO high content screening system (Molecular Devices). Cell fluorescence was quantified using the accompanying software. Three biological replicates were prepared for each group. For determination of ROS, N-acetyl-cysteine (ENZO Life Science, Farmingdale, NY), a ROS scavenger, was used to cardiomyocytes at the concentration of 0.5 mM for 1 h prior to sorafenib administration.

Statistical analysis. Results are expressed as mean ± standard error. Differences among groups were tested for statistical significance using the unpaired Student’s t-test, and P < .05 was considered significant. For multiple comparisons, one-way analysis of variance followed by the Tukey-Kramer procedure was used. All statistical analyses were conducted using Statcel 2 software (OMS, Tokyo, Japan).

RESULTS

Sorafenib Cardiotoxicity in Zebrafish line MK009
To assess the cardiotoxicity of sorafenib in zebrafish, we created a novel zebrafish line MK009 by crossing nacre and SAG4A. nacre is a zebrafish mutant with a pigmentless body due to lack of the mitfa gene (Lister et al., 1999). SAG4A is a transgenic zebrafish in which GFP is selectively expressed in the heart (Kawakami et al., 2004). MK009 is an attractive model for cardiac imaging, because the fluorescent heart can be clearly observed in the pigmentless body (Supplementary Figure S1A).
The maximum plasma concentration of sorafenib after a 28-day cycle in patients receiving 400 mg, 2 times per day was 8.5 μM and the trough concentration was 6.4 μM in phase 1 clinical trials (Cheng et al., 2011; Strumberg et al., 2007; Tolcher et al., 2010). Thus, in the following experiments we choose the concentrations of 1 and 3 μM. The survival rate of zebrafish treated with 3 μM sorafenib was decreased by ~20% at 5 dpf (Figure 1A, black solid line). In addition, zebrafish exposed to 3 μM sorafenib showed some noticeable body malformations including a curved body shape, and an uninflated swim bladder (Figure 1B). No zebrafish were alive in the 3 μM sorafenib group on 6 dpf. Zebrafish treated with 1 μM sorafenib did not show any decrease in survival rate until 6 dpf (Figure 1A, gray solid line) or any external malformation (Figure 1B) until 5 dpf.

We then assessed the cardiac function in zebrafish treated with 1 μM sorafenib using the quantitative fluorescent cardiac imaging that we have developed (Umemoto et al., 2013) (Supplementary Figure S1B). As 3 μM sorafenib caused significant mortality by 120 hpf, 1 μM sorafenib was used for the remaining experiments. At 96 hpf (24-h exposure), both long VDd and VDs in zebrafish treated with sorafenib were significantly (P < .05) shorter than those of the control group (Figure 2A). However, the calculated EF and FS were not significantly different between zebrafish with sorafenib treatment and those of control group (Figure 2B). At 120 hpf (48 h exposure), long VDs in zebrafish treated with sorafenib were significantly (P < .01) longer than those of the control group (Figure 2C).

Both the calculated EF and FS in zebrafish with sorafenib treatment were significantly (P < .01) decreased compared with the control group (Figure 2D). These results suggest that 1 μM sorafenib treatment from 72 to 96 hpf induced selectivity longitudinal impairment with preserved both EF and FS of zebrafish ventricle. Sorafenib Reduces Expression of stc1 in Heart of MK009

To clarify the molecular mechanism of sorafenib-induced cardiotoxicity by eliminating the secondary effect of ventricular dysfunction, we performed transcriptome analysis of hearts isolated from zebrafish with or without exposure to 1 μM sorafenib for 24 h. Using a commercially available DNA microarray system, we analyzed the expression of 21,383 probes that were reliably expressed in the hearts (GSE 61155) and identified 215 genes significantly (false discovery rate; FDR < 10%) dysregulated between hearts with and without sorafenib treatment (Supplementary Table S2). In total, 56 and 159 genes were significantly down- and upregulated, respectively, in the heart with sorafenib treatment.

To identify genes that might be relevant to sorafenib-induced cardiotoxicity, we performed a bioinformatics approach using the 215 genes dysregulated by sorafenib treatment in MK009 and Pathway Studio, a bioinformatics software. First, we selected “heart failure” as a functional entity in the Pathway Studio and identified 32 genes that were linked to heart failure (Supplementary Figure S2A). It has been shown that ERK (MAPK1 and MAPK3) are involved in sorafenib-induced cardiotoxicity (Cheng et al., 2011; Hasinoff and Patel, 2010). Therefore, we selected “MAPK1” and “MAPK3” as molecular entities in the Pathway Studio and identified 14 genes whose expression was regulated by both MAPK1 (ERK2) and MAPK3 (ERK1) (Supplementary Figure S2B). Two and 12 genes were down- and upregulated in the hearts with sorafenib treatment, respectively. It has been reported that sorafenib inhibits the phosphorylation of MAPK1 and MAPK3 and the function of these ERKs. We focused on stc1 because the expression of stc1 was positively regulated by ERK (Nguyen et al., 2009) and VEGF (Holmes and Zachary, 2008) signaling. We performed qPCR to confirm the downregulation of stc1 in the hearts of zebrafish exposed to sorafenib. As shown in Figure 3A, the expression level of stc1 was significantly (P < .05) lower than that of the control heart.

We performed qPCR to confirm the downregulation of STC1 exposed to 0.3 μM sorafenib or vehicle for 24 h. As shown in Figure 3B, the expression level of STC1 in human cardiomyocytes exposed to sorafenib was significantly (P < .05) lower than that exposed to the vehicle.

Reduction of stc1 Impairs Cardiac Function in MK009

We examined whether the reduction of stc1 affected cardiac function in MK009. To knock down stc1 expression, zebrafish embryos were injected with stc1 MO. We conducted stc1 knockdown using splicing inhibitor MO, and confirmed the gene knockdown level by qPCR. (P < .01 sorafenib vs vehicle, Figure 3C). Functional cardiac imaging at 96 hpf revealed that both long VDs and long VDd in stc1 morphant (Figure 3D, black bar) were significantly shorter than those in control zebrafish that were injected with control MO (Figure 3D, white bar). The calculated EF and FS were not significantly different between stc1 morphant and control zebrafish (Figure 3E). The reduction of both long VDs and long VDd in stc1 morphant at 96 hpf was similar to that in zebrafish exposed to sorafenib from 72 to 96 hpf (Figure 2A).
To confirm whether the decrease in long VDs and long VDd in stc1 morphant was caused by reduced expression of stc1, zebrafish embryos were co-injected with stc1 MO and stc1 mRNA. stc1 MO was designed to bind to the junction of the first intron and second exon of stc1, thus stc1 MO was not likely to bind to stc1 mRNA. Therefore, stc1 mRNA should be translated into stc1 protein without interference from stc1 MO. Functional cardiac imaging at 96 hpf revealed that both long VDs and long VDd were significantly increased in zebrafish co-injected with stc1 MO and stc1 mRNA (Figure 3D, gray bar) compared with those of stc1 morphant (Figure 3D black bar). The calculated EF and FS were not statistically different among the three groups (Figure 3E). These results suggest that reduction of stc1 could cause dysregulation of ventricular dimensions in zebrafish.

**Sorafenib-Induced STC1 Reduction Causes ROS Overproduction in Human Cardiomyocytes**

It has been reported that sorafenib increases ROS generation in human cancer cells (Coriat et al., 2012; Fumarola et al., 2013) and that STC1 suppresses ROS generation induced by angiotensin II in rodent cardiomyocytes (Liu et al., 2012). To examine the relationship between stc1 and cardiotoxicity, we observed accumulation of ROS throughout the body of zebrafish treated with 1 μM sorafenib for 2 h (Figure 4A). ROS were detected in vivo by adapting a protocol based on DHE. ROS generation of zebrafish treated with sorafenib was significantly increased in vehicle-treated zebrafish (Figure 4B). NAC, a known scavenger of ROS, inhibited the increase in ROS level by sorafenib treatment (Figure 4B). To examine whether reduction of stc1 affected ROS generation, zebrafish embryos were injected with 0.3 mM stc1 MO or 0.3 mM control MO (Figure 4C). ROS generation was significantly increased in zebrafish injected with stc1 MO compared with control MO (Figure 4D and E) and with stc1 MO + stc1 mRNA (Figure 4E).

Furthermore, to validate the involvement of STC1 in sorafenib-induced cardiotoxicity in humans, we conducted an in vitro study using human cardiomyocytes. To examine whether sorafenib induces ROS generation in cardiomyocytes, we treated human cardiomyocytes with or without 0.3 μM sorafenib for 1 h and assessed the ROS generation using fluorescent imaging. As shown in Figure 5A and B, ROS generation was significantly increased in human cardiomyocytes exposed to sorafenib compared with that in the control. To examine whether reduction of STC1 could affect ROS generation, human cardiomyocytes were transfected with STC1 siRNA. ROS generation was significantly increased in human cardiomyocytes transfected with STC1 siRNA compared with NC siRNA (Figure 5C and D). As shown in Figure 5E, the expression of STC1 in human cardiomyocytes transfected with STC1 siRNA were significantly lower than that of NC siRNA.

**Forced Expression of stc1 Reverses Sorafenib-Induced Cardiotoxicity**

We investigated whether forced expression of stc1 reversed sorafenib-induced cardiotoxicity in zebrafish. Zebrafish embryos were injected with or without stc1 mRNA and treated with or without 1 μM sorafenib from 72 to 96 hpf. Functional cardiac imaging at 96 hpf revealed that both long VDs and long VDd in zebrafish without stc1 mRNA injection and with sorafenib treatment (Figure 6A, black bar) were significantly shorter than that in zebrafish with stc1 mRNA injection and without sorafenib treatment (Figure 6A, white bar). These results suggest that forced expression of stc1 can reverse the cardiotoxicity induced by sorafenib.

**FIG. 2.** Sorafenib cardiotoxicity in MK009 zebrafish. MK009 were treated with or without 1 μM sorafenib from 72 to 96 or 120 hours post-fertilization (hpf). Functional cardiac imaging was performed at 96 or 120 hpf. A, Assessment of ventricular end-systolic dimension (VDs) or ventricular end-diastolic dimension (VDd) of both short and long axes at 96 hpf. B, Calculated ejection fraction (EF) and fractional shortening (FS) based on VDd and VDs at 96 hpf. A, B, control, n = 9; sorafenib, n = 13; ***P < .01. C, Assessment of VDs or VDd of both short and long axes at 120 hpf. D, Calculated EF and FS based on VDd and VDs at 120 hpf. C, D, control, n = 10; sorafenib, n = 12; ***P < .01.
those in zebrafish without stc1 mRNA and sorafenib (Figure 6A, white bar) and zebrafish with stc1 mRNA and without sorafenib (Figure 6A, gray bar). Both long VDs and long VDd in zebrafish with stc1 mRNA injection and sorafenib treatment (Figure 6A, shaded bar) did not differ significantly from those in zebrafish without stc1 mRNA and sorafenib (Figure 6A, white bar). There was no significant difference in the calculated EF and FS among the 4 groups (Figure 6B). Furthermore, forced expression of stc1 mRNA suppressed sorafenib-induced ROS generation (Figure 6C). These results suggest that reduction of stc1 could be responsible for sorafenib-induced cardiotoxicity.

DISCUSSION

The pigmentless zebrafish line MK009 was developed to allow quantitative observation of heart function that is highlighted by GFP. Owing to fluorescent cardiac image analysis of MK009, using a high-content imager with LED illumination as an inverted fluorescent microscope, we were able to demonstrate that the ventricular dimension of the longitudinal axis decreased with preserved EF and FS in zebrafish treated with 1 μM sorafenib from 72 to 96 hpf. This cardiac functional imaging is an innovative way to detect clearly the target tissues, if the intensity of fluorescence in the tissues is as weak as that of cardiac GFP in MK009, because the LED illumination of the high-content imager is brighter than the halogen illumination of simple manual microscopes. We also found that the longitudinal dimension increased with reduced EF and FS in zebrafish treated with 1 μM sorafenib from 72 to 120 hpf (Figure 2D). These results were consistent with a previous study that reported a significant increase in the end-systolic dimension of both ventricular axes and decreased FS after administration of 0.5 μM sorafenib from 48 to 120 hpf (Cheng et al., 2011). In a previous clinical study, a reduction in the ventricular dimension of the longitudinal axis was detected in patients with early-stage heart failure induced by sorafenib (Di Salvo et al., 2011). This is in good agreement with our study that showed reduced longitudinal

FIG. 3. Reduction of stc1 expression caused a significant decrease in ventricular dimension. A, B, quantitative polymerase chain reaction (qPCR) to assess stc1 (STC1) expression. A, MK009 were treated with or without 1 μM sorafenib from 72 to 96 hours post-fertilization (hpf). The hearts were isolated from these zebrafish and qPCR was performed to assess the expression of stc1. * P < .05, zebrafish treated with sorafenib versus zebrafish without sorafenib treatment. Four samples per group were analyzed. One sample contained 20 hearts of zebrafish. B, Human cardiomyocytes were treated with or without 0.3 μM sorafenib for 24h. qPCR was performed to assess the expression of STC1. * P < .05. Four samples per group were analyzed. C, MK009 at 1- to 4-cell stages were injected with 0.3 mM stc1 morpholino oligonucleotides (MOs) and raised to 96 hpf. Total RNA was extracted from the whole body and qPCR was performed to assess expression of stc1. ** P < .01, zebrafish injected with stc1 MO versus zebrafish injected with control MO. Eight zebrafish per group were analyzed. D, E, Assessment of cardiac function in zebrafish injected with control MO, stc1 MO or stc1 MO and stc1 mRNA. Twenty zebrafish per group were analyzed. For multiple comparisons, one-way analysis of variance followed by the Tukey-Kramer procedure was used. D, Assessment of ventricular end-systolic dimension (VDs) or ventricular end-diastolic dimension (VDd) of both short and long axes. E, Calculated ejection fraction (EF) and fractional shortening (FS) based on VDd and VDs. * P < .05
dimension in zebrafish treated with sorafenib from 72 to 96 hpf. These results suggest that the zebrafish cardiotoxic model treated with 1 μM sorafenib for 24 h showed selective longitudinal ventricular dysfunction with preserved EF and FS, which mimicked sorafenib-induced cardiotoxicity in humans.

To establish the mechanism of cardiotoxicity as on-target and off-target effects, we aimed to identify differentially expressed genes in the sorafenib-induced cardiotoxic model. Sorafenib inhibited RAF1, which is coded by mitogen activated protein kinase kinase kinase (MAP3K). Once activated, RAF1 can phosphorylate the dual specificity protein kinases MEK1/2 to activate them. These in turn phosphorylate the serine/threonine-specific protein kinases ERK1/2 to activate them (Roux and Blenis, 2004). Activated ERKs are effectors of cell physiology and control gene expression involved in the regulation of cell cycle progression, proliferation, cytokinesis, transcription,
differentiation, senescence, and apoptosis (Orphanos et al., 2009). RAF1 inhibits two proapoptotic kinases, apoptosis signal-regulating kinase (ASK)1 and mammalian sterile 20-like kinase (MST)2, which are important in oxidative-stress-induced injury. Deletion of RAF1 gene in the heart leads to a dilated, hypokinetic heart with increased cardiomyocyte apoptosis (Feng et al., 2013).

In this study, to determine an accurate gene expression profile in the sorafenib-induced cardiotoxic model, we performed transcriptome analysis using a cDNA microarray from a small amount of heart tissue (20 hearts per group). In a previous transcriptome analysis of heart tissue in zebrafish (Burns and MacRae, 2006), the authors used 500 isolated hearts of zebrafish larvae for each condition of their microarray experiment. However, their technology for heart isolation using a micropipette carries a risk of including noncardiac tissue in the samples. In our study, we developed a method to isolate cardiac tissue easily from zebrafish larvae by using microsurgical scissors and preparing RNA samples extracted from 20 hearts for each DNA chip. We analyzed the expression data using Gene Set Enrichment Analysis (GSEA) by Pathway Studio to find gene ontology (GO) associated with the 215 genes whose expression...
levels were altered by sorafenib treatment in zebrafish. From the DNA microarray analysis, we identified 14 genes whose expression was regulated by both MAPK1 and MAPK3, and was linked to heart failure using a bioinformatics approach of Pathway Studio. Two and 12 genes were down- and upregulated in the hearts with sorafenib treatment, respectively (Supplementary Figure S2B).

We focused on oxidative stress as a potential off-target effect of sorafenib. It is known that sorafenib can provoke rapid generation of various ROS in two HCC cell lines (HepG2 and Hepa 1.6) and human umbilical vein endothelial cells, and exerts dose-dependent cytostatic and cytotoxic effects (Chiou et al., 2009; Jacob et al., 2002). Additionally, it is recognized that anticancer agents, such as adriamycin, induce dose-limiting cardiotoxicity. There is a consensus that this toxicity is related to the induction of ROS. Induction of ROS in the heart by adriamycin occurs via redox cycling of the drug at complex I of the electron transport chain (Berthiaume and Wallace, 2007). We hypothesized that sorafenib might induce the overproduction of ROS in the heart. In fact, Hasinoff and Patel (2010) reported that sorafenib treatment of neonatal rat cardiomyocytes caused dose-dependent damage and increased oxidative activity at therapeutically relevant concentrations. A previous clinical study also reported that serum levels of advanced oxidation protein products on ROS in patients with HCC treated with sorafenib were increased after 15 days treatment (Coriat et al., 2012). However, these authors could not clarify the mechanism of sorafenib-induced cardiotoxicity. As a result of the GSEA in our study, STC1 was classified in “ROS generation” in the GO list of cell processing categories (Supplementary Table S3). To the best of our knowledge, this is the potential mechanism of sorafenib-induced cardiotoxicity. Here, we demonstrated overproduction of ROS and reduction of STC1 in zebrafish treated with 1 μM sorafenib for 2 and 24 h, respectively. Overproduction of ROS and reduction of STC1 in human cardiomyocytes treated with 0.3 μM sorafenib for 1 h were also shown in our study. We also observed a cytotoxic phenotype in human cardiomyocytes treated with 1 μM sorafenib for 24 h (data not shown). Moreover, STC1 knockdown

FIG. 6. Forced expression of stc1 mRNA reversed sorafenib-induced cardiotoxicity. A, B, MK009 zebrafish at 1- to 4-cell stages were injected with or without stc1 mRNA and treated with or without 1 μM sorafenib from 72 to 96 hpf. Functional cardiac imaging was performed at 96 hpf. A, Assessment of VDs or VDd of both short and long axes. B, Calculated ejection fraction (EF) and fractional shortening (FS) based on ventricular end-systolic dimension (VDs) and ventricular end-diastolic dimension (VDd). Seventeen zebrafish per group were analyzed. *P < .05, **P < .01. C, Quantitative analysis of reactive oxygen species (ROS) in the zebrafish. MK009 zebrafish at 1- to 4-cell stages were injected with or without stc1 mRNA and treated with or without 1 μM sorafenib from 72 to 74 hpf. Thirteen zebrafish per group were analyzed. *P < .05, **P < .01. A, B, C, For multiple comparisons, one-way analysis of variance followed by the Tukey-Kramer procedure was used.
in zebrafish and human cardiomyocytes significantly increased ROS production compared with the cardiomyocytes treated with vehicle. Our results suggest that induction of ROS by sorafenib in zebrafish and human cardiomyocytes is associated with reduction of STC1.

STC1 is a glycoprotein hormone involved in calcium/phosphate homeostasis, which was originally discovered as a secretory hormone of the corpus of Stannius, an endocrine gland of teleosts (jiang et al., 2000). This gene is evolutionarily highly converged, with the first 202 amino acids showing 90% similarity between human STC1 (NM-003155) and zebrafish stc1 (NM-001045457). Consequently, the molecular function is also highly conserved in vertebrates, including fish (Ishibashi and Imai, 2002). In addition, mammalian STC1 is widely expressed in various tissues including the heart (Guo et al., 2013; Yeung et al., 2012), similar to zebrafish in this study. Clinically, STC1 is upregulated in the hearts of patients with myocardial infarction and idiopathic dilated cardiomyopathy (Guo et al., 2013). However, it remains unclear whether downregulation of STC1 by sorafenib treatment is associated with ROS generation in cardiomyocytes as a mechanism of cardiotoxicity of sorafenib.

Liu et al. have reported that human STC1 activates a novel antioxidant pathway in cardiomyocytes through induction of uncoupling protein 3 UCP3, suggesting that STC1 plays an important role in suppressing ROS in the heart (Liu et al., 2012). In our study, reduction of STC1 activated ROS production in human cardiomyocytes. Nguyen et al. also have reported that the expression of stc1 is positively regulated by ERK signaling (Nguyen et al., 2009). Therefore, sorafenib might induce overproduction of ROS caused by STC1 downregulation, through inhibition of ERK signaling, resulting in cytotoxic effects in both zebrafish heart and human cardiomyocytes.

Finally, we performed forced expression of stc1 to rescue the ventricular dysfunction in zebrafish treated with sorafenib. To normalize expression level of stc1 in zebrafish treated with sorafenib, we injected stc1 mRNA into zebrafish eggs and then treated them with 1 μM sorafenib from 72 to 96 hpf. We found that the forced expression of stc1 in zebrafish with sorafenib treatment normalized the decreasing longitudinal ventricular dimensions caused by sorafenib treatment. Consequently, these studies suggest that STC1 plays a role in protection against oxidative stress by ROS overproduction in cardiomyocytes following sorafenib treatment.

In conclusion, we established that STC1 downregulation is responsible for sorafenib-induced cardiotoxicity through activated ROS generation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

This work was supported in part by grants from Mie University Graduate School of Medicine; the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; the Japan Science and Technology Agency; the Long-range Research Initiative of the Japan Chemical Industrial Association (2010NT02, 2011NT02-01); the Johns Hopkins Center for Alternatives to Animal Testing (2011-08, 2012-17); and the Sumitomo Foundation (113498).

ACKNOWLEDGMENTS

We express our gratitude to Dr K. Dohi and Dr K. Miyashita for helpful discussions, and T. Oka for teaching us bioinformatics analysis. We are grateful to Dr K. Kawakami (National Institute of Genetics, Japan) for providing the SAC44a line. We thank R. Kawase, S. Ichikawa, M. Ariyoshi, H. Nakayama and J. Koiwa for zebrafish breeding, and R. Ikeyama and Y. Tamura for secretarial assistance.

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