Supplemental Material

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 (H_2O_2) (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 [1]. 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 [2].

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 [3]. 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), H_2O_2 (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 dH_2O) 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:

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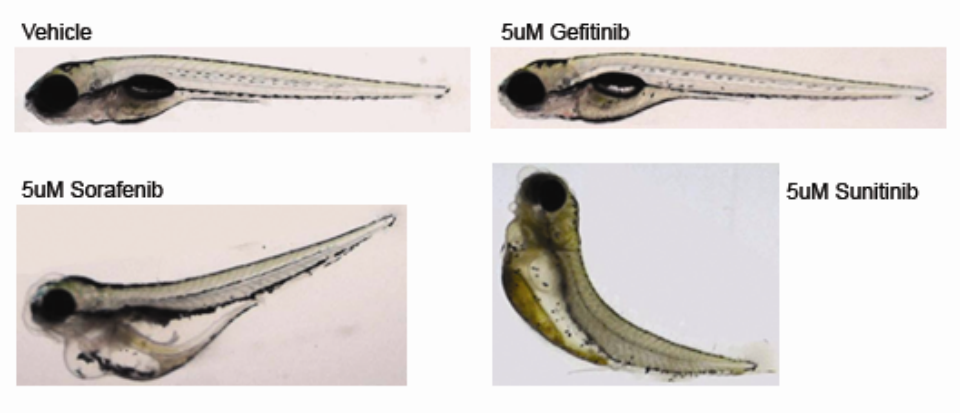
Supplemental Figures and Legends:

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.