Supplemental Material

Supplemental Methods

Cell culture, transfection, and treatment

Human cardiac fibroblasts (HCFs, Promega) were cultured in fibroblast basal medium FBM-3 supplemented with 10% fetal bovine serum (FBS), supplements (Promega), 100 µg/ml penicillin and 100 µg/ml streptomycin under standard cell culture conditions (37°C, 5% CO_2). Murine HL-1 cardiomyocytes (Louisiana State University Health Sciences Center, New Orleans, LA) were cultured in Claycomb medium supplemented with 10% FBS (Sigma-Aldrich) as described (Claycomb, Lanson et al. 1998). Human pulmonary fibroblasts (HPFs, Promega) were cultured in fibroblast growth medium 2 kit (Promega) supplemented with 2% FBS and 100 µg/ml penicillin and 100 µg/ml streptomycin, and NRK49Fs in DMEM low Glucose (Lonza, Basel, Switzerland) supplemented with 10 % FBS and 100 µg/ml penicillin and 100 µg/ml streptomycin, under standard cell culture conditions (37°C, 5% CO_2). Neonatal rat cardiomyocytes (NRCMs) were kept in MEM with 5 % FBS at 37°C, 1% CO_2. Transfection was carried out with specific miRNA mimics, inhibitors or negative controls (mirVana® miRNA mimics/inhibitors, Applied Biosystems) at a final concentration of 30nM. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Treatment with natural compounds or DMSO control was performed at concentrations as indicated in the figure legends for 24h. Stimulation with angiotensin II was done at final concentrations of 1 µM or 1 mM and renewed after 24h for additional 24h.

Animal experiments

All animal studies involving mice were performed in accordance with the relevant guidelines and regulations and with the approval of the Niedersächsisches Landesamt für

Verbraucherschutz und Lebensmittelsicherheit (LAVES, Germany). To induce systemic hypertension, subcutaneous implantation of osmotic minipumps (ALZET®) delivering angiotensin II (Ang II) at 3 mg/kg/day was performed in male C57BL/6N wild type mice (Charles River Laboratories, Sulzfeld, Germany) at age of 8 - 10 weeks. The operation was performed after intraperitoneal injection of 4 mg/kg xylazine and 100 mg/kg ketamine. An osmotic minipump infusing Ang II was implanted for 14 days (ALZET micro-osmotic pump model 1002) in preventive study and for 56 days (re-implantation with ALZET micro-osmotic pump model 1004; 28 days) in therapeutic study. The natural compounds (dissolved in DMSO) or the solvent alone were injected intraperitoneally every other day until the endpoint, starting one day or two weeks after start of Ang II-infusion for the preventive or the therapeutic study, respectively. Bufalin was injected at a dose of 0.5 mg/kg and lycorine at 4.5 mg/kg. At two or eight weeks after the surgery, as indicated in the figure captions, cardiac function was assessed by echocardiography (Vevo2100, Fujifilm/VisualSonics, Canada) and subsequent hemodynamic measurements (PVR-1045 Pressure Volume catheter Millar Instruments; MPVS Ultra Foundation System/LabChart 7, ADInstuments Ltd, UK), both under general anesthesia. General anesthesia was initiated with isoflurane (5% isoflurane in 100% oxygen, using an oxygen-flow of 5 l/min) in an induction chamber. Maintenance of anesthesia for echocardiography was achieved using 0.8-2% isoflurane in 100% oxygen, using an oxygen-flow of 0,8 l/min and a breathing mask; and for hemodynamic measurements with 1-2% isoflurane in 100% oxygen via a breathing mask followed by tracheal respiration. Animals were euthanized at endpoint and hearts were removed for histological and biochemical analysis and liver and kidneys were collected for toxicological evaluation.

Male Dahl salt-sensitive rats (SS/JrHsdMcwiCrl; 5 weeks) were obtained from Charles River (USA). The experimental protocol was approved by the ethics committee of the Brigham and Women's Hospital and by the Ethical Committee for Animal Experimentation of the University of Navarra (Spain) and conducted according to both animal care and use committee guidelines. Animals were housed in a climate-controlled facility with a 12-hour/12-hour

light/dark cycle and provided free access to food and water. A group of 39 animals started receiving a high salt diet (8%) at 7 weeks of age. For drug treatment, the hypertensive rats with high salt diet were divided into 3-sub groups, DMSO control group (High salt diet control, n=16), bufalin treated group (Bufalin, n=10) and lycorine treated group (Lycorine, n=13). A control group of 17 animals was fed with a normal diet (Normal diet control). After 2 weeks of high salt diet, the Bufalin group was treated with 0.1 mg/kg of bufalin and the lycorine group was treated with 4.5 mg/kg of lycorine. High salt diet control groups were injected with saline as vehicle every other day. An echocardiographic study (Vevo 3100; Fujifilm/Visualsonics, Canada) was performed before starting the diet (week 6), at 14 weeks and, together with hemodynamic analysis (SPR-847 Pressure Volume catheter Millar Instruments; MPVS Ultra Foundation System/LabChart 7, ADInstuments Ltd, UK) at the endpoint of experiment.

RNA isolation

Total RNA of tissues, fractionated and cultured cardiac cells was isolated using RNeasy Mini Kit (Qiagen) or TriFast method (Peqlab) according to the manufacturer's instructions. Subsequent quantification and quality control was performed with Synergy HT Reader (BioTek).

qRT-PCR analysis

For quantitative detection of mRNAs, reverse transcription of 100-1000 ng total RNA prior to real-time qPCR was performed using the iScript Select cDNA synthesis kit (Bio-Rad), following the manufacturer's instructions. Real-time qPCR was performed in a CFX96 Touch™ Real-Time PCR Detection System (Biorad, Hercules, USA) using specific primers (**Supplemental Table 1**) and the iQ SYBR Green Mix (Bio-Rad) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or 18S ribosomal RNA (18S rRNA) was used as housekeeping control for gene-specific expression levels.

For miRNAs quantification, specific TaqMan MicroRNA Assays (**Supplemental Table 1**) were used following the manufacturer's protocol using 50 ng total RNA. miRNAs were quantified with the ViiA™ 7 Real-Time PCR System (Life Technologies, Carlsbad, USA) and miRNA levels were normalized to the small RNA molecule snoRNA-202 for mouse samples and to RNU48 for human samples.

Proliferation Assay

Bromodeoxyuridine (BrdU) Cell Proliferation ELISA Kit (Cell Proliferation ELISA, BrdU, colorimetric, Roche) was used to measure the proliferation rate of HCFs, HPFs, NRKFs or HL1 cells according to the manufacturer's instructions.

AnnexinV-7AAD-staining

For differentiation between viable, apoptotic and necrotic cells FlowColletTM Annexin Red Kit (Millipore) was used according to the manufacturer´s instructions 24h after treatment of HCFs with bufalin, gitoxigenin, lycorin, anisomycin or geldanamycin at effective doses. Externalization of phosphatidylserine to the cell surface of early apoptotic cells was followed by specific binding of fluorescently labelled Annexin-V to phosphatidylserine. 7-Aminoactinomycin (7-AAD), a membrane in permeant dead cell dye was used to counterstain late apoptotic cells with disrupted membrane integrity as well as necrotic cells exhibiting leaky membranes but no phosphatidylserine externalisation. The fluorescence intensity for 7-AAD and Annexin-V for each single cell was analysed using guava easyCyteTM Flow Cytometer (Merck-Millipore, Darmstadt, Germany).

MicroRNA target prediction

The microRNA databases and target prediction tools miRBase (http://microrna.sanger.ac.uk/), PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/index.html) were used to screen for potential microRNA targets.

MicroRNA profiling

Global miRNA profiling was performed in primary HCFs 24h after treatment with effective doses of the lead compounds. Total RNA was isolated using the miRNeasy Mini Kit (Qiagen), quantified, integrity- and quality-assessed by on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany) (triplicates from 3 independent experiments were pooled per group). The RNA sequencing library was generated from 0.5-1µg of total RNA using TruSeq® Small RNA Library Prep Kits v2 (Illumina) according to manufacturer's protocols. The libraries were sequenced on Illumina HiSeq2500 using TruSeq SBS Kit v3-HS (50 cycles, single ended run) with an average of 5-10 x106 reads per RNA sample. Raw data (FASTQ files) were applied to OASIS 1.0 sRNA Detection pipeline.[1] Briefly, FASTQ files were trimmed with cutadapt 1.7.1 (http://code.google.com/p/cutadapt/) removing Illumina adapter sequences (TGGAATTCTCGGGTGCCAAGG) and nucleotides with phred scores below 20. The min-max limit for sequence length was set to 15-32nt. Alignment of trimmed FASTQ sequences was done with STAR Aligner [2] against the following databases: Mirbase version 20; release date: 2013-10-1, snRNA: Ensembl: Human s. version GRCh38.74; snoRNAEnsembl: Human s. version GRCh38.74; rRNA: Ensembl: Human s. version GRCh38.74. The number of mismatches allowed for successful alignment was set to 5% of sequence length. Counts per smallRNA (feature) were calculated using featureCounts.[3] Differential expression of smallRNA was determined by DESeq2.[4] Cluster 3.0 was used for hierarchical clustering to present top-50 significantly deregulated miRNAs in a heatmap.

Protein extraction and Western-Blotting

Cells were lysed using lysis buffer supplemented with protease-blocker Pefabloc SC. Proteins were separated according to their mass via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes which were subsequently blocked with 5 % milk in Tris-buffered-saline-Tween (TBS-T). Primary antibody was incubated overnight in 5 % milk in TBS-T for Collagen type 1 (Sigma Aldrich, #HPA008405) or selenoprotein P1 (Abcam, #ab109514). Binding of secondary antibody conjugated with horseradish peroxidase (HRP) against rabbit (Cell Signaling Technology, #7074) was visualized using chemiluminescence, and quantified using ImageJ software. Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, #ab8245) was used as an internal loading control and for normalization of protein quantification.

Measurement of extracellular collagen content

HCFs were cultured under standard conditions as described above. After 24 hours, medium was changed to 1ml FBM-3 supplemented with 1% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin per well of a 6-well plate. Cells were cultured in this medium for three consecutive days and for the last 24 hours, bufalin or lycorine were directly added to the medium at final concentrations of 1µM or 5µM, respectively. Supernatants were subjected to Sircol assay (Biocolor) according to manufacturer instructions.

Neonatal rat cardiomyocyte size measurement

NRCMs were isolated as described previously [5] and stimulated with 40ng/ml AngII. After 24 hours, AngII stimulation was renewed and NRCMs treated with 1µM bufalin or 5µM lycorine.

Cells were cultured in 48 well plates for additional 24 hours with 1% CO_2. Immunofluorescent staining was performed using anti α-sarcomeric actinin (Sigma A7811) and Alexa Fluor 594 antibodies (Invitrogen).

Immunofluorescent staining of HCFs

HCFs were seeded into sterile coverslips in 24-well plates, cultivated as described above and treated with 1μM bufalin or 5μM lycorine for 24 hours. Immunofluorescent staining was performed using anti prolyl 4-hydroxylase (P4HA1) (Novus Biologicals) and Alexa Fluor 488 antibodies (Invitrogen).

Histology and microscopy

For histological assessment of cardiac fibrosis in mice, paraffin embedded sections of the LV were stained with Picro-sirius red and the collagen content calculated as the percentage of fibrotic areas in the heart. Quantifications of microscopic images were performed with NIS-elements BR 3.2 package (Nikon Instruments Inc.) and Adobe Photoshop CS3 extended. For murine cardiomyocyte size measurement, cardiomyocyte cell membranes of the myocardium were visualized by wheat germ agglutinin stain coupled to Alexa Fluor 488 (Invitrogen). The area of cardiomyocytes was calculated using the NIS-Elements BR 3.2 package (Nikon Instruments Inc.). TUNEL was performed in cryo-sections of murine hearts after infusion of AngII for eight weeks using the In Situ Cell Death Detection Kit Fluorescein (Sigma-Aldrich). For quantification of apoptotic cells, TUNEL- and DAPI-positive cells were counted in each image.

Histological evaluation in the Dahls rat was performed blinded to the experimental group. Myocardial samples were immediately fixed in 4% buffered formalin, embedded in paraffin, and serially sectioned in 4 mm thick sections. Sections were stained with collagen-specific Picro-sirius red (Sirius red F3BA in aqueous picric acid). The fraction of myocardial tissue

occupied by collagen fibers or collagen volume fraction (CVF) was determined as a percentage by quantitative morphometry with an automated image analysis system (CellˆD, Olympus Soft imaging Solutions GmbH, Germany). Sections were analyzed under an automatized microscope, and all the fields covering myocardial tissue section were digitized. Stained collagen areas were segmented by interactive gray-level thresholding of shading-corrected images, and then the subendocardial regions were interactively discarded.

Cardiomyocyte area in rat hearts was measured in cross-sectionally oriented cardiomyocytes with a visible central nucleus located in the subendocardial part of the myocardium in picrosirius red-stained sections. A minimum of 50 cardiomyocytes was analyzed in each section.

Pulmonary edema was evaluated histologically in hematoxylin-eosin-stained sections.

For histopathological analysis of kidney and liver, paraffin embedded sections of the organs were stained with Periodic Acid-Schiff (PAS) and Haematoxilin Eosin (HE), respectively.

Cardiac collagen quantification

Histological tissue sections were digitalized with a tissue slide scanner (Axio Scan.Z1, Zeiss, Germany) at a 20 x magnification. Automated image analysis was performed with tissuemorph DP software (Visiopharm, Horsholm, Denmark) to detect collagen in histological PSR images as described previously [6] with some modifications. In brief, a threshold for the grayscale image of the green channel band was set from 0 to 140 in combination with the grayscale image of the red-green contrast band (red color band vs. green color band) from 50 to 256 to detect collagen content. The heart tissue was detected in grayscale image of the red color band without values of the grayscale image of the blue channel band from 180 to 256. Prior to automated tissue analysis, an image random sampling was performed to collect approximately 40 images at 10 x magnification. For the assessment of regional fibrosis, we assigned vessel areas as region of interest (ROI) and the rest part of heart as other tissue in

each randomly acquired image. The percentage of collagen was calculated by dividing the detected collagen area by the total tissue area either in the ROI (defined as perivascular fibrosis) or in the other tissue (depicted as interstitial fibrosis). Sum of both perivascular and interstitial fibrosis is presented as total fibrosis.

Collagen cross-linking

To evaluate the degree of collagen cross-linking in hearts of the Dahls rat, the amount of cross-linked (insoluble) and non-cross-linked (soluble) collagen were determined as previously described [7] using two colorimetric assays: Fast Green-Sirius Red to obtain total collagen and Sircol-based assay to obtain soluble collagen. The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen.

Echocardiography

Transthoracic echocardiography in mice was performed using Vevo 2100/3100 (VisualSonics). Briefly, mice were anaesthetized initially with 5% of isoflurane and fixed to a platform at 37 °C. Recordings were taken from the Parasternal Long Axis View (PLAX) and Parasternal Short Axis View (PSAX) of the left ventricle (LV) both in B and M modes. Mitral valve flow and mitral valve annulus velocities were evaluated using pulsed-wave and tissue Doppler imaging from the apical four chamber view, respectively. Echo data was analysed using the Vevo LAB software (VisualSonics). All measurements were averaged from three cardiac cycles per animal at comparable heart rates. To assess wall global longitudinal strain (GLS) and regional peak longitudinal strains of the heart we used the VevoStrain software (VisualSonics). Myocardial performance index (MPI) is a Doppler echocardiographic parameter and calculated by sum of the isovolumic contraction and relaxation times (ICT and IRT) divided by the ejection time (ET).

Echocardiography in rats was performed using a Vevo 3100 ultrasound system (Visualsonics, Toronto, Canada) equipped with a real time micro-visualization scan head probe (RMV-710 B) working at a frame rate ranging between 110 and 120 frames per sec (fps). A nosepiece-transducer with a central frequency of 25 MHz, a focal length of 15 mm and 70 mm of nominal spatial resolution was used. Rats were anesthetized with isoflurane, at a concentration of 4% (induction) and 2% (maintenance) in 100% oxygen. Each animal was placed on a heating table in a supine position. Trans mitral Doppler was used for the assessment of diastolic parameters.

Hemodynamic pressure–volume measurements

For hemodynamic measurements, mice were initially anesthetized with 5% isoflurane and mechanically ventilated (MiniVent respirator Harvard Apparatus, Holliston, MA). Pressure-volume (PV) measurements were acquired using a 1F microtip pressure-volume catheter (PVR 1045; Millar Instruments, Houston, TX) coupled with a Powerlab/4SPacquisition system (AD Instruments Ltd, Oxford, UK) under constant maintenance of 37° C. PV loops were analyzed with Labchart 7 AD Instruments Ltd, Oxford, UK). All volume-based parameters were calibrated to the cardiac volumes acquired by echocardiography and all measures averaged from ten - fifteen consecutive beats. After baseline measurements, transient occlusion of the inferior vena cava was performed to assess ventricular compliance via the end-diastolic pressure–volume relationship (EDPVR) and end systolic pressure volume relationship (ESPVR).

All pressure volume loop data were analyzed using a cardiac pressure–volume analysis program (LabChart 8, ADInstruments), and the heart rate (HR), maximal left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), maximal slope of systolic pressure increment (+dP/dt) and diastolic decrement (–dP/dt), and the time-constant

of isovolumic relaxation (Tau) using the Weiss method were computed. All hemodynamic parameters were calculated and corrected according to in vivo volume calibrations based on echocardiographic end systolic volume and end diastolic volume data. These parameters were also determined under conditions of changing preload, elicited by transiently compressing the inferior vena cava in ventilated anesthetized animals following thoracotomy. Since +dP/dt may be preload-dependent, in these animals pressure–volume loops recorded at different preloads were used to derive other useful indices that may be less influenced by loading conditions and cardiac mass. These measures include the end systolic pressure volume relationship (ESPVR_linear) and end diastolic pressure volume relationship (EDPVR_linear).

Fractionation of cardiac cells

Fractionation of cardiac cells was performed as previously described. C57BL/6N mice were anaesthetized with isoflurane and injected with 100 µl heparin in 0,9% NaCl. After cannulation of the aorta and excision of the heart, retrograde perfusion was performed for 3 minutes inside and additional 3 minutes outside the mouse with pre-warmed perfusion buffer (113 mM NaCl, 4,7 mM KCl, 0,6 mM KH_2PO_4, 0,6 mM Na_2HPO_4, 1,2 mM $MgSO_4$-$7H_2O$, 0.032 mM Phenol Red, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM HEPES, 30 mM Taurine, 0.1%Glucose, 10 mM 2,3-Butanedione monoxime) followed by pre-warmed digestion buffer (113 mM NaCl, 4,7 mM KCl, 0,6 mM KH_2PO_4, 0,6 mM Na_2HPO_4, 1,2 mM $MgSO_4$-$7H_2O$, 0.032 mM Phenol Red, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM HEPES, 30 mM Taurine, 0.1%Glucose, 10 mM 2,3-Butanedione monoxime, 12.5 µM $CaCl_2$, 700 U/ml Collagenase II) for 10 - 20 minutes. Cardiac ventricles were further dissociated mechanically by cutting and shearing in digestion buffer. Collagenase II digestion was stopped by diluting the cell suspension with stop buffer (113 mM NaCl, 4,7 mM KCl, 0,6 mM KH_2PO_4, 0,6 mM Na_2HPO_4, 1,2 mM $MgSO_4$-$7H_2O$, 0.032 mM Phenol Red, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM HEPES, 30 mM Taurine, 0.1%Glucose, 10 mM 2,3-Butanedione monoxime, 12.5 µM

CaCl2, 10% FBS). The obtained cell suspension was filtered through a 100 µm cell strainer and further diluted with AMCF medium containing 1% FBS (10.8 g MEM, 10% NaHCO3, 2ng/ml vitamin B12, 1X Penicillin/streptomycin). The appearance of rod-shaped beating cardiomyocytes was assessed under a light microscope, followed by sedimentation of cells for 10 minutes on ice. Sedimented pellets were washed in PBS, centrifuged for 5 minutes at 3000 rpm at 4°C, frozen in liquid nitrogen and stored at -80°C. The remaining supernatants were centrifuged for 3 minutes at 30g at RT to remove residual cardiomyocytes. The cardiomyocyte-free supernatants were further centrifuged for 5 minutes at 1500 rpm at RT. Pellets containing cardiac fibroblasts were resuspended in AMCF medium containing 1% FBS and pre-plated for 1 hour on a 10 cm petri dish in a 1% CO2 incubator. The attached cells were washed with PBS, harvested in PBS with a cell scraper, centrifuged at 3000 rpm for 5 minutes at 4°C, frozen in liquid nitrogen and stored at -80°C. The remaining pre-plating medium, containing non-attached endothelial cells, was centrifuged at 1500 rpm for 5 minutes at 4°C and the resulting pellet suspended in MACS buffer (MACS bovine serum albumin stock solution diluted 1:20 in autoMACS rinsing solution, both from Miltenyi Biotec). CD146 MACS beads (Miltenyi Biotec) were added and the samples were incubated for 15 minutes at 4°C. Afterwards MACS buffer was added again and the samples were centrifuged at 1500 rpm for 5 minutes at 4°C. The resulting pellets were dissolved in MACS buffer and the suspension was applied on a MACS separating column. After 3 washing steps with MACS buffer, columns were removed from the magnetic field and endothelial cells were collected in MACS buffer. Following centrifugation at 3000 rpm for 5 minutes at 4 °C, pellets were frozen in liquid nitrogen and stored at -80 °C.

Blood pressure measurements

For mean systolic and diastolic blood pressure measurements, mice were implanted with miniature subcutaneous radiotelemetry devices (Data Sciences International, La Jolla, Calif) under anaesthesia. Blood pressure was measured continuously in freely moving AngII-

infused mice injected with bufalin, lycorine or with the solvent only, maintained on a 12:12 h day:night cycle with constant access to food and water.

Plasma sampling and biochemical analysis

EDTA-plasma samples were drawn from the mice and centrifuged at 400g for 10 min. The supernatant was stored at −80°C until analysis. Enzyme activities were determined on a Modular P800 automatic analyzer using standard methods (Roche Diagnostics): Creatinine, glutamate-oxaloacetate-transaminase (GOT) and glutamate-pyruvate-transaminase (GPT). Urea was measured photometrical (UREAL; kinetic urease test with glutamate dehydrogenase (GLDH) on a Cobas 6000 analyzer system (Roche Diagnostics).

Quantification of bufalin and lycorine by liquid chromatography (LC) and quadrupole time-of-flight mass spectrometry (QTOF-MS). Natural compounds-analysis was performed on >50mg cardiac whole-tissue lysates and on 50µl EDTA-plasma of mice 9 min after intraperitoneal injection of the compounds. Both were snap-frozen in liquid nitrogen and stored at −80 °C until extraction. After addition of extraction solution (Acetonitrile/Methanol/Wasser (2/2/1, v/v/v) for tissue and Acetonitrile/Methanol (1/1, v/v) for EDTA-plasma) and disrupting of tissue, samples were boiled at 95°C for 15 min and subsequently cooled on ice. The samples were frozen overnight at -20°C and centrifuged for 10 min at 4°C and 20.800 x g. Supernatants were collected and frozen overnight at -20°C. After thawing, the samples were centrifuged for 10 min at 20800 x g (4°C). The supernatant fluid was transferred to a 2 mL reaction tube and evaporated under a gentle nitrogen stream at 40°C. The residual pellet was reconstituted in 75 µL of HPLC grade water (J.T. Baker., Deventer, The Netherlands) containing 2 µM pirfenidone (internal standard for bufalin) and 100 ng/ml tenofovir (internal standard for lycorine). This solution was centrifuged for 10 min at 20800 x g (4°C) and the supernatant fluid was transferred to an MS vial. Aliquots of 10 µL were injected into the LC-QTOF system. Reversed Phase chromatographic separation of

lycorine and bufalin was performed using a Shimadzu HPLC-system (Shimadzu, Duisburg, Germany), consisting of two HPLC-Pumps (LC-30AD), a temperature controlled autosampler (SIL-30AC), a degasser (DGU-20A5) and a column oven (CTO-20AC). For chromatographic analysis a C18 reversed phase column (ZORBAX Eclipse XDB-C18 1.8 µ, 50 x 4,6 mm; Waters, Milford, Massachusetts, USA) was used. This column was connected to a C18-Security guard (Phenomenex, Aschaffenburg, Germany) and to a 2 µ column saver (Sigma-Aldrich; St. Louis, Missouri, USA). The column was maintained at 30°C. Solvents were water (solvent A; J.T. Baker., Deventer, The Netherlands) and methanol (solvent B; J.T. Baker., Deventer, The Netherlands), each containing 0.1% formic acid. The initial solvent composition was set to 95 % A and 5% B. A gradient was applied to achieve separation of lycorine and bufalin. In a first step solvent B was increased to 95 % within 14 min. This solvent composition was kept for 1 min before the column was reequilibrated to starting conditions for 6 min. Total analysis runtime was 21 min. A flow rate of 0.4 mL/min was used. Detection and quantification of lycorine and bufalin was carried out on a QTOF mass spectrometer (QTOF5600; Sciex, Framingham, Massachusetts, USA) equipped with an electrospray ionization source, operating in positive ionization mode. For analyte detection, the following mass ranges (+/- 20 ppm of the protonated ion) were used: lycorine: m/z 288.1172 – 288.1288 Da, bufalin: m/z 387.2453 – 387.2607 Da, tenofovir (IS for lycorine): m/z 288.0875 – 288.0913 Da and pirfenidone (IS for bufalin): 186.0875 – 186.095 Da. Control of LC and QTOF as well as data sampling was performed by Analyst software TF (version 1.5.1., Sciex). Calibration curves were created by plotting peak area ratios of lycorine and bufalin, respectively, and the corresponding internal standard versus the nominal concentration of the calibrators. Both calibration curves were calculated using quadratic regression and 1/x weighing.

Supplemental Tables

Supplemental Table 1: qPCR primers and TaqMan miRNA detection assays used in this study.

qPCR primers (mRNA)		
Name	**Forward primer (5'->3')**	**Reverse primer (5'->3')**
hsa-18S rRNA	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
mmu-18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
mmu-ß-Actin	ATCAAGATCATTGCTCCTCCTG	AGGGTGTAAAACGCAGCTCA
hsa-GAPDH	CCAGGCGCCCAATACG	CCACATCGCTCAGACACCAT
hsa-IL-6	TAGCCGCCCCACACAGACAG	GGCTGGCATTTGTGGTTGGG
hsa-IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC
hsa-α-SMA	CCTGACTGAGCGTGGCTATT	GATGAAGGATGGCTGGAACA
mmu-IL-1ß	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG
mmu-TNFα	TACTGAACTTCGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC
mmu-BAX	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
mmu-BCL2	ATAACGGAGGCTGGGTAGGT	CCAGAATCCACTCACACCCC
hsa-COL1A1	QuantiTect Primer Assay: QT00037793 (Qiagen, Hilden Germany)	
hsa-CTGF	QuantiTect Primer Assay: QT00052898 (Qiagen, Hilden Germany)	
mmu-CDR1	QuantiTect Primer Assay: QT02324539 (Qiagen, Hilden Germany)	
mmu-CHPF2	QuantiTect Primer Assay: QT00014973 (Qiagen, Hilden Germany)	
mmu-SEPP1	QuantiTect Primer Assay: QT00252301 (Qiagen, Hilden Germany)	
hsa-SEPP1	QuantiTect Primer Assay: QT01008175 (Qiagen, Hilden Germany)	

TaqMan assays (miRNA)	
Name	
RNU48	TaqMan Assay: 001006 (Applied Biosystems, Foster City, CA, USA)
mmu-snoRNA-202	TaqMan Assay: 001232 (Applied Biosystems, Foster City, CA, USA)
hsa-miR-671-5p	TaqMan Assay: 197646_mat (Applied Biosystems, Foster City, CA, USA)

A Preventive study

DMSO control — Vehicle | DMSO control | Bufalin | Lycorine — Angiotensin II

500 μm

B Perivascular fibrosis [%] — DMSO control Vehicle, DMSO control Bufalin Lycorine Angiotensin II — ns, *, *

C Interstitial fibrosis [%] — DMSO control Vehicle, DMSO control Bufalin Lycorine Angiotensin II — *, *, 0.06

D Substance/heart tissue [nmol/g] — Bufalin, Lycorine

E Plasma concentration [μM] — Bufalin, Lycorine

F SBP [mmHg] — AngII+DMSO, AngII+bufalin, AngII+lycorine — time (days)

G DBP [mmHg] — AngII+DMSO, AngII+bufalin, AngII+lycorine — time (days)

H TUNEL DAPI — DMSO control Vehicle | DMSO control | Bufalin | Lycorine — Angiotensin II — 1mm

TUNEL/DAPI — DMSO control Vehicle, DMSO control Bufalin Lycorine Angiotensin II — ns, ns, ns

I BAX/BCL2 — DMSO control Vehicle, DMSO control Bufalin Lycorine Angiotensin II — ns, ns, ns

J TNFalpha/18S rRNA FC of vehicle control — DMSO control Vehicle, DMSO control Bufalin Lycorine Angiotensin II — ns, ns, ns

K IL-1beta18S rRNA FC of vehicle control — DMSO control Vehicle, DMSO control Bufalin Lycorine Angiotensin II — ns, ns, ns

A

Creatinine [µmol/l] — Urea [mmol/l] — GOT [U/l] — GPT [U/l]

DMSO control (Vehicle), DMSO control, Bufalin, Lycorine (Angiotensin II)

B

Vehicle: DMSO control, Bufalin, Lycorine

Angiotensin II: DMSO control, Bufalin, Lycorine

1 mm

C

Cardiomyocyte size [µm2]

ns

ns

*

DMSO — Bufalin — Lycorine

D

DMSO — Bufalin — Lycorine

Vehicle

WGA
DAPI

Angiotensin II

200µm

A Therapeutic study

DMSO control | DMSO control | Bufalin | Lycorine

Vehicle | Angiotensin II

500 µm

B

Perivascular fibrosis [%]

DMSO control Vehicle | DMSO control | Bufalin | Lycorine
Angiotensin II

C

Interstital fibrosis [%]

DMSO control Vehicle | DMSO control | Bufalin | Lycorine
Angiotensin II

D

ESPVR

DMSO | Bufalin | Lycorine

□ Vehicle
■ AngiotensinII

E

DMSO | Bufalin | Lycorine

Vehicle

PW doppler

Tissue doppler

Angiotensin II

F

G

H

A Mouse therapeutic study

Kidney PAS

Liver H&E

DMSO control — Vehicle

DMSO control — Angiotensin II

Bufalin — Angiotensin II

Lycorine — Angiotensin II

200 µm

200 µm

B

I.p. injection of bufalin or lycorine

d 0   d 14   d 56

Implantation of angiotensin II minipumps

Assessment of cardiac function and fibrosis

C

ns

ns

ns

Fibrosis [%] FC of vehicle

DMSO control (Vehicle), DMSO control, Bufalin, Lycorine (Angiotensin II)

DMSO control — Vehicle

DMSO control — Angiotensin II

Bufalin — Angiotensin II

Lycorine — Angiotensin II

500 µm

D

ns

ns

ns

MPI

DMSO control (Vehicle), DMSO control, Bufalin, Lycorine (Angiotensin II)

E

***

**

*

IVRT/AET

DMSO control (Vehicle), DMSO control, Bufalin, Lycorine (Angiotensin II)

Supplemental Figures

Supplemental Fig. 1: *In vitro* **assessment of collagen secretion and fibroblast-specific proliferative actions of bufalin and lycorine. (A)** HCFs treated with bufalin release 50% of the amount of collagen secreted in the DMSO control group (one-way ANOVA, n=3). Bufalin and lycorine act fibroblast-specifically as evidenced by no impact on hypertrophic growth of neonatal rat cardiomyocytes induced by AngII, depicted in **(B)** representative images and **(C)** quantification of cell size measurement (one-way ANOVA, Tukey's multiple comparisons test, n=21-100). Cardiomyocytes were stained with α-actinin (red) and nuclei with DAPI (blue) after 24h treatment with natural compounds as indicated and 48h of stimulation with Ang II; scale bar: 100 μm. Dose-dependency of proliferation-inhibitory effects of lycorine was evident **(D)** in the rat renal fibroblast cell line NRK49F, whereas bufalin potently inhibited the proliferation of **(E)** primary human pulmonary fibroblasts (HPFs). The cells were treated with the compounds for 24h as indicated and proliferation of respective fibroblasts was measured by BrdU-ELISA (one-way ANOVA, Dunnett's multiple comparisons test, n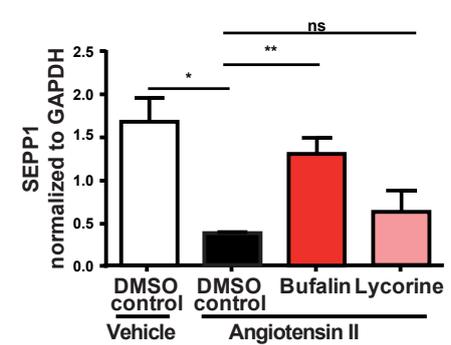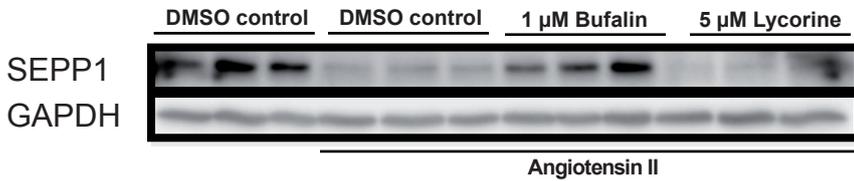=3). **(F)** Reduced body weight (BW) upon Ang II infusion compared to control animals and comparable BW by treatments compared to Ang II infustion as indicated (one-way ANOVA, Tukey's multiple comparisons test, n = 14/17/17/14).

All values from **(A)** and **(C)** – **(F)** are represented as mean±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

HPFs = human pulmonary fibroblasts

Supplemental Fig. 2: Cardiac uptake of bufalin and lycorine and exclusion of cardio-protective effects due to prevention of hypertension, cardiomyocyte apoptosis or inflammation. (A) 2 weeks of bufalin treatment in preventive approach significantly reduced Ang II-induced fibrosis in both perivascular **(B)** and interstitial region **(C)**. Lycorine treatment for 2 weeks also showed anti-fibrotic effect on interstitial fibrosis with a borderline of significance **(A -C)** (one-way ANOVA, Tukey's multiple comparisons test, n = 10/11/10/11). Effective uptake of bufalin and particularly lycorine into the heart, as evidenced by **(D)** myocardial substance amounts; also **(E)** levels in the plasma after i.p. injections at the respective effective doses are shown (n = 3/3). Time-course of telemetric **(F)** systolic and **(G)** diastolic blood pressure values (means of 24 hours) confirms induction of systemic hypertension upon AngII-infusion (started on day 0), which remains elevated upon injection with bufalin or lycorine (two-way repeated measures ANOVA, n = 3/5/4). **(H)** AngII-infusion for eight weeks does not induce apoptosis in the myocardium (one-way ANOVA, Tukey's multiple comparisons test, n = 6/7/7/7). DNA-fragmentation was visualized by terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL)-staining (green), and nuclei were stained with DAPI (blue); scale bar: 1 mm. **(I)** Quantification of mRNA levels of *BAX* and *BCL2* in the murine cardiac tissues of the therapeutic approach show a comparable *BAX/BCL2* ratio within all groups tested (one-way ANOVA, Tukey's multiple comparisons test, n = 12/9/10/7). Quantification of mRNA levels of TNFα **(J)** and IL-1β **(K)** in murine cardiac tissues after Ang II infusion for two weeks show no significant changes among tested groups as indicated (one-way ANOVA, Tukey's multiple comparisons test, n = 10/11/10/11).

All values from **(A)** – **(C)** are represented as mean±SEM. *p<0.05; scale bar: 500 μm. All values from **(D)** – **(E)** are represented as mean+min to max**.** All values from **(F)** – **(G)** are represented as mean of 24h±SEM. All values from **(H)** – **(K)** are represented as mean±SEM.

SBP = systolic blood pressure; DBP diastolic blood pressure; *BAX = BCL2 associated X*; *BCL2 = B-cell lymphoma 2*; *TNFα = tumour necrosis factor alpha*; *IL-1β = interleukin-1β.*

Supplemental Fig. 3: Bufalin and lycorine prevented global fibrosis in Ang II induced mice of therapeutic approach without affecting cardiomyocyte size. (A) Overall, levels of markers of kidney (creatinine and urea) and liver (glutamate-oxaloacetate-transaminase, GOT and glutamate-pyruvate-transaminase, GPT) damage remain unchanged upon treatment of mice with bufalin or lycorine for two weeks. **(B)** Representative images of cardiac fibrosis in whole heart sections of therapeutic study. Both compounds significantly ameliorate cardiac fibrosis in established diastolic heart failure by 50%; scale bar: 1 mm. No significant change in cardiomyocyte size shown in representative images **(D)** and corresponding quantification **(C)** from mice treated with bufalin or lycorine as compared to control animals of the therapeutic approach (two-way ANOVA, Tukey's multiple comparisons test, n=12/8/7/12/14/10). Cardiomyocyte cell membranes were stained with wheat germ agglutinin (WGA) (green) and nuclei with DAPI (blue); scale bar: 200 µm. All values from **(C)** are represented as mean±SEM. *$p<0.05$.

GOT = glutamate-oxaloacetate-transaminase; GPT = glutamate-pyruvate-transaminase

Supplemental Fig. 4: Bufalin and lycorine prevented regional fibrosis in Ang II induced mice of therapeutic approach and improved diastolic function in hypertensive mice of therapeutic approach. The anti-fibrotic effect of compounds on regional fibrosis has been recaptured in therapeutic approach after 6 weeks of treatments with compounds as well **(A)**. Significant reduction in perivascular **(B)** and interstitial fibrosis **(C)** by treatment with bufalin. Partial prevention in perivascular fibrosis **(B)** and significant reduction in interstitial fibrosis **(C)** by treatment with lycorine have been shown (one-way ANOVA, Tukey's multiple comparisons test, 19/8/7/17/18/16) scale bar: 500 µm. **(D)** No impact on Ang II induced elevation in ESPVR by compounds treatment (two-way ANOVA, Tukey's multiple comparisons test, n=19/8/7/17/18/16). **(E)** Representative images of PW Doppler and tissue doppler velocity. Lead compounds recover velocity of tissue motion during diastole (decreased MPI and E/A ratio as compared to AngII solvent only control indicated by arrow)

elevated by AngII-infusion. **(F)** Systolic velocity is measured with a speckle tracking trace of the LV long-axis 6 segments and corresponding representative images. Significant prevention and amelioration of the AngII-caused downshift of both radial **(G)** and longitudinal strain rate **(H)** in the anterior basal (AB), mid anterior (AM), anterior apex (AA), posterior apex (PA), mid posterior (PM) and posterior basal (PB) regions (Table 2) of the heart upon treatment with bufalin and lycorine (two-way ANOVA, Tukey's multiple comparisons test, n = 17/6/7/12/16/16). Radial strain (%) **(G)**, longitudinal strain (%) **(H)**.

All values from (A) – (C) are represented as mean±SEM. *p<0.05; ***p<0.001; All values from (D) and (G) – (H) are represented as mean±SEM. *p<0.05.

ESPVR = end systolic pressure-volume relationship; PW = pulsed wave; AA = anterior apex; AB = anterior base; AM = mid anterior; EF = ejection fraction; PA = posterior apex; PB = posterior basal; other abbreviations as in Fig. 1

Supplemental Fig. 5: Administration of bufalin or lycorine has no effect on kidney or liver in rodents in therapeutic studies and less fibrosis induction by Ang II in female mice, which is still ameliorated by lead compounds. (A) PAS-staining of kidneys (upper panel) and H&E of livers (lower panel) of mice after eight weeks of AngII-infusion and six weeks treatment with bufalin or lycorine, starting two weeks after the implantation of the minipumps, showing no change in morphology, fibrosis, necrosis and inflammation; scale bar: 200 µm. **(B)** Therapeutic *in vivo* study using female mice. Bufalin, lycorine or DMSO were injected intraperitoneally every other day for six consecutive weeks starting two weeks after the implantation of minipumps filled with AngII. **(C)** Bufalin and lycorine counteract further progression of cardiac fibrosis, even though collagen deposition in the myocardium following AngII-infusion is less strongly induced in female compared with male mice (one-way ANOVA, Tukey's multiple comparisons test, n = 7/6/5/6), scale bar 500 µm. **(D)** No significant differences of MPI among groups tested (one-way ANOVA, Tukey's multiple comparisons test, n = 7/5/5/6). **(E)** Significant deterioration of diastolic parameter IVRT/AET

by AngII-infusion in female mice treated with the solvent only (controls), which seems less pronounced in mice treated with bufalin or lycorine (one-way ANOVA, Tukey's multiple comparisons test, n = 7/5/5/6).

All values from **(C)** – **(E)** are represented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001.

Abbreviations as in **Fig. 1** and **2**.

Supplemental Fig. 6: Lycorine and bufalin prevent the development of cardiac fibrosis and diastolic dysfunction in hypertensive rats with no effect on kindney, liver and lung as well as food intake. (A) No impact on E to E' ratio has been shown neither by high salt diet nor by treatment with compounds (one-way ANOVA, Tukey's multiple comparisons test, n = 8/7/7/9). **(B)** PAS-staining of kidneys (upper panel) and H&E of livers (lower panel) of rats after eight weeks of high salt diet and six weeks treatment with compounds, starting two weeks after commencing the high salt diet. After eight weeks of high salt diet, kidneys show moderate inflammation, severe diffuse tubular injury, and severe loss of brush border, numerous protein- and detritus-containing casts, thrombotic microangiopathy, focal and segmental as well as global glomerulosclerosis, and severe arterio- and arteriolosclerosis, which remains unchanged after lycorine treatment. Livers show loss of glycogen and steatosis in the hypertensive rat, independently of lycorine treatment. Additionally, vessel wall necrosis and thrombotic microangiopathy is evident in animals that did not receive lycorine; scale bar: 200 µm. No significant impact of the lead compounds on pulmonary and cardiac congestion, evidenced by normal lung weights **(D)** and heart weight **(E)** normalized to body weight (one-way ANOVA, Tukey's multiple comparisons test, n = 7/9/1013) and a throughout absence of edema, visualized by hematoxylin-eosin-stained sections of the lung **(C)**; scale bar: 200 µm. **(F)** Lycorine has no impact on weekly high salt diet intake of Dahls salt sensitive rats (n = 9/12).

All values from **(A)** and **(D)** – **(F)** are represented as mean±SEM. *p<0.05.

Supplemental Fig. 7: Characterization of miR-671-5p and the direct target selenoprotein P1. (A) No impact on fibrosis markers *α-SMA, CTGF* and pro-inflammatory cytokines *IL-6* and *IL-8* in primary HCFs after inhibition of miR-671-5p (unpaired t-test, n=4-8). **(B)** Bufalin, gitoxigenin, lycorin, anisomycin and geldanamycin do not directly drive transcription of the host gene of miR-671-5p, shown by measurement of *Chondroitin Polymerizing Factor 2 (CHPF2)* expression in primary HCFs upon treatment as indicated compared to control (one-way ANOVA, Tukey's multiple comparisons test,, n=3). **(C)** MiR-671-5p levels in murine fibroblast fractions after infusion with angiotensin II for 2 weeks and simultaneous treatment with bufalin (one-way ANOVA, Tukey's multiple comparisons test, n = 6/10/5). **(D)** miR-671-5p is ubiquitously expressed in various cell types (n=3 per group) and murine organs (n=6 per group) as indicated. **(E)** Effective silencing of *SEPP1* in HCFs (unpaired t-test, n=6) leads to **(F)** increased COL1A1 expression in HCFs as shown in a representative Western Blot. **(G)** Decrease of SEPP1 protein levels in HCFs after AngII-stimulation is reversed in particular by bufalin (one-way ANOVA, n = 3). **(H)** In line, in vivo heart samples recaptured the trend that AngII induced reduction in cardiac protein expression of SEPP1 is rescued by bufalin treatment (two-way ANOVA, n = 4).

All values from **(A)** – **(E)** and **(H)** are represented as mean±SEM. *$p<0.05$; **$p<0.01$;; values from **(F)** are represented as mean±SD.

CHPF2 = Chondroitin Polymerizing Factor 2; HUVEC = human umbilical vein endothelial cells; HCF = human cardiac fibroblasts; HL-1 = cell line from a mouse atrial cardiomyocyte tumor lineage; other abbreviations as in **Fig. 1, 5** and **6.**

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