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Natural Compound Library Screening Identifies New Molecules for the Treatment of Cardiac Fibrosis and Diastolic Dysfunction

Running Title: Katharina et al.; Anti-Fibrotic Compounds in Diastolic Dysfunction

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Abstract

Background: Myocardial fibrosis is a hallmark of cardiac remodeling and functionally involved in heart failure (HF) development, a leading cause of deaths worldwide. Clinically there is no therapeutic strategy available that specifically attenuates maladaptive responses of cardiac fibroblasts, the effector cells of fibrosis in the heart. Therefore, we aimed at the development of novel anti-fibrotic therapeutics based on natural-derived substance library screens for the treatment of cardiac fibrosis.

Methods: Anti-fibrotic drug candidates were identified by functional screening of 480 chemically diverse natural compounds in primary human cardiac fibroblasts (HCFs), subsequent validation and mechanistic in vitro and in vivo studies. Hits were analyzed for dose-dependent inhibition of proliferation of HCFs, for modulation of apoptosis and extracellular matrix expression. In vitro findings were confirmed in vivo, using an angiotensin II (Ang II)-mediated murine model of cardiac fibrosis in both preventive and therapeutic settings, as well as in the Dahl salt sensitive rat model. To investigate the mechanism underlying the anti-fibrotic potential of the lead compounds, treatment-dependent changes in the noncoding RNAome in primary HCFs were analyzed by RNA-deep sequencing.

Results: High-throughput natural compound library screening identified 15 substances with anti-proliferative effects in HCFs. Using multiple in vitro fibrosis assays and stringent selection algorithms we identified the steroid bufalin (from Chinese toad venom) and the alkaloid lycorine (from Amaryllidaceae species) to be effective anti-fibrotic molecules both in vitro and in vivo leading to improvement in diastolic function in two hypertension-dependent rodent models of cardiac fibrosis. Administration at effective doses did not change plasma damage markers nor the morphology of kidney and liver, providing first toxicological safety data. By next-generation sequencing we identified the conserved microRNA (miR) miR-671-5p and downstream the anti-fibrotic selenoprotein P1 (SEPP1) as common effectors of the anti-fibrotic compounds.

Conclusions: We identified the molecules bufalin and lycorine as drug candidates for therapeutic applications in cardiac fibrosis and diastolic dysfunction.

Key Words: Diastolic dysfunction; cardiac fibrosis; hypertension; bufalin; lycorine; miR-671-5p

Non-Standard Abbreviations and Acronyms
HF    heart failure
HCF   human cardiac fibroblasts
Ang II angiotensin II
COL1A1 collagen, type I, alpha 1
HPF   human pulmonary fibroblasts
DT    deceleration time
MPI   myocardial performance index
EDPVR end diastolic pressure volume relationship
BAX   bcl-2-associated X protein
BCL2  b-cell lymphoma 2
TNFα  tumor necrosis factor alpha
IL-1β Interleukin 1 beta
IVRT  isovolumic relaxation time
AET   aortic ejection time
CVD   cardiovascular disease
Clinical Perspective

What is new?

- Natural compound library screening in human cardiac fibroblasts identified novel compounds with anti-fibrotic properties.
- The compounds bufalin and lycorine show highly potent anti-fibrotic effects in vitro and in vivo and improve diastolic function in relevant models of heart failure.
- We provide evidence for cardioprotective, anti-fibrotic effects of the natural compounds bufalin and lycorine with a promising safety-tolerability profile setting the ground for prospective preclinical and clinical studies with the aim to specifically treat cardiac fibrosis and diastolic dysfunction.

What are the clinical implications?

- Although some state of the art medications may improve fibrosis and diastolic dysfunction, currently no therapeutic strategy is available that is specifically designed to target fibroblasts, the effector cells of fibrosis in the heart.
- We provide strong evidence of natural compound-based preventive and therapeutic strategies against the development of cardiac fibrosis and diastolic dysfunction in clinically relevant models of heart failure.
- Preclinical and clinical development of specific cardiac anti-fibrotic molecules as presented here could lead to transformational changes in the treatment of patients with cardiac fibrosis and diastolic dysfunction.
Introduction

In the healthy heart extracellular matrix is essential to maintain both structure and integrity of the organ. In contrast, cardiac stress results in excessive production of extracellular matrix leading to cardiac fibrosis.\(^1\),\(^2\) Fibrosis subsequently triggers myocardial stiffness, ultimately impairing left ventricular diastolic and later systolic function.\(^3\) Myocardial fibrosis favors progression to end-stage HF, arrhythmias and ischemia and therefore correlates with an increased mortality in patients, even under state-of-the-art treatment.\(^4\) Thus, cardiac fibrosis not only constitutes a major determinant for the clinical outcome of HF patients but also a therapeutic target of utmost interest and importance.

We thus here applied a natural-derived substance library screen to search for novel anti-fibrotic, cardio-protective lead compounds for further development into new anti-fibrotic therapeutics. Subsequent refining strategies of functional in vitro and in vivo studies uncovered the cardiac glycoside bufalin and the alkaloid lycorine. Both molecules potently repressed the fibrotic response in human cardiac fibroblasts (HCFs) and both prevented and reversed fibrosis in cardiac fibrosis mouse models (implanted osmotic minipumps for systemic angiotensin II-infusion). Additionally, inhibition of cardiac fibrosis along with improvement in diastolic performance could be confirmed in the Dahl salt sensitive rat model of HF with diastolic dysfunction. Previously, these two identified drug candidates were discovered to exhibit anti-tumor activities in pre-clinical studies.\(^5\)-\(^16\) Moreover, neuroprotective, anti-inflammatory, anti-osteoporosis as well as anti-microbial effects of the compounds have been described.\(^7\),\(^12\),\(^17\)-\(^20\)

However, application of bufalin or lycorine in the prevention or treatment of cardiac fibrosis and diastolic dysfunction has never been explored. Also, their mode of action or influence on non-coding RNAs involved in cardiac fibrosis is unknown.
Methods

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), as well as with that of the Brigham and Women’s Hospital and by the Ethical Committee for Animal Experimentation of the University of Navarra (Spain).

Statistical analysis

All in vitro experiments were performed 3 or more independent times, as indicated in the figure captions; in duplicate, triplicate or quadruplicate wells. Independent number of animals in each group is indicated in the figure legends. Data are displayed as mean of independent experiments/independent animal samples ± SEM, if not otherwise specified in the figure legend. Statistical analysis was carried out using the GraphPad Prism 6 software (GraphPad Prism Software Inc. San Diego, California, USA). For comparison of two groups, unpaired two-tailed Student’s t-test was applied and for analysis of three or more groups, one-way ANOVA with Turkey’s and two-way ANOVA with Tukey’s multiple comparisons test was performed, if not otherwise specified in the figure legend. In all cases, a p-value of 0.05 or lower was considered statistically significant.

A detailed method section is available in the Online Data Supplement.

The data and analytic methods will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Study materials only can be provided upon signing a MTA and if still available.
Results

Natural compound screening identifies bufalin and lycorine as specific anti-fibrotic molecules in human cardiac fibroblasts

Excessive accumulation of extracellular matrix in the diseased heart is caused by an increase in both proliferation rates and collagen production of cardiac fibroblasts. To uncover natural compounds suppressing fibrotic responses in cardiac fibroblasts, proliferation of HCFs was studied after incubation with 480 individual compounds, selected from a database comprising 150,000 natural products for a maximum of chemical diversity, using OptiSim algorithm (Fig. 1A - B). Applying a selection threshold of fibroblast proliferation inhibition FC (fold change) ≥ 75%, we identified 15 candidates, of which 5 inhibited proliferation rates in a dose-dependent manner (Fig. 1B - C). Next, we excluded cell death to be involved in the observed decrease in proliferation triggered by these compounds (bufalin, gitoxigenin, lycorine, anisomycin and geldanamycin). Therefore, HCFs were treated with respective compounds using the effective doses and subjected to FACS-analysis after annexin-7AAD-staining (Fig. 1D). To confirm fibroblast-specificity of the remaining candidates, potential effects on proliferation rates of the cardiomyocyte cell line HL-1 were studied. As depicted in Fig. 1A, with exception of anisomycine, all tested substances acted specifically on cardiac fibroblasts only, as evidenced by no impact on the proliferation of HL-1 cells (Fig. 1E). Based on these selection criteria, the cardiac glycoside bufalin and the alkaloid lycorine were further assayed for suppression of COL1A1-expression, the rigid and highly cross-linked extracellular matrix component produced in excess by activated fibroblasts. Importantly, treatment of HCFs with the two lead compounds, and in particular with bufalin, effectively decreased fibroblast COL1A1 protein levels (Fig. 1F).
Likewise, collagen amounts secreted into the supernatant of HCFs were reduced by bufalin (Supplemental Fig. 1A).

To further elaborate on fibroblast-specific actions of bufalin and lycorine, we determined the cell size of neonatal rat cardiomyocytes stimulated with Ang II upon treatment with the two lead drug candidates. Importantly, neither bufalin nor lycorine counteracted the hypertrophic growth of cardiomyocytes in vitro stimulated by Ang II (Supplemental Fig. 1B, C). Of note, lycorine, but not bufalin exerted slight inhibitory effects on proliferation of the rat renal fibroblast cell line NRK49F (Supplemental Fig. 1D). In contrast, particularly bufalin inhibited proliferation of primary human pulmonary fibroblasts (HPFs), (Supplemental Fig. 1E). These results might suggest a potential therapeutic implication of the substances not only in cardiac, but also in renal, or pulmonary fibrotic diseases, respectively.

**Bufalin and lycorine prevent fibrosis and preserve cardiac function in angiotensin II-induced cardiac remodelling**

To verify whether the identified anti-fibrotic substances prevent fibrosis-development at early stages also in vivo, C57BL/6N mice were infused with Ang II by subcutaneously implanted minipumps for two weeks. The natural substances (dissolved in DMSO) or the solvent alone (as control) were injected intraperitoneally every other day until the endpoint, commencing one day after start of Ang II-infusion. Histological sections of the heart were studied for fibrosis-development and cardiac function was assessed both echocardiographically and hemodynamically. A schematic representation of the preventive in vivo study is depicted in Fig. 2A. Ang II-infusion led to a significant decrease in body weight (Supplemental Fig. 1F) which linked with increased heart-to-body weight ratios in animals infused with the solvent only (controls); however, bufalin or lycorine did not significantly prevent this effect (Fig. 2B).
Treatment with bufalin significantly prevented cardiac fibrosis as shown by a reduction of collagen-deposition in histological sections of the hearts (Fig. 2C). Lycorine showed also global anti-fibrotic effects, although this was of borderline significance (Fig. 2C). However, further assessment of regional fibrosis measurements showed significant reductions in interstitial fibrosis as well as in perivascular fibrosis by treatment with bufalin. Treatment with lycorine also prominently decreased interstitial fibrosis, however significance of this effect was borderline (Supplemental Fig. 2A - C).

Fibrosis-development is associated with diastolic and eventually progression to systolic dysfunction in hypertensive mice, hereby closely recapitulating pathological remodeling common in fibrotic cardiac disease in humans. Thus, we evaluated mitral inflow and Tissue Doppler signals to assess the effect of the compounds on diastolic function (Fig. 2D). Echocardiographic evaluation of diastolic function revealed early signs of diastolic dysfunction with impaired relaxation after 2 weeks of Ang II treatment (Figure 2 D-H) with decreased E/A, increased DT, accompanied by slower peak mitral annular velocity, E’. Both compounds conferred improvements of relaxation, as evidenced by a decrease of the Myocardial Performance Index (MPI) and the deceleration time (DT), as well as rescued E to A ratios and E’. The increase of E’ indicates true improvement of tissue function, making pseudo-normalization unlikely (Fig. 2H). The preventive effect of lycorine on MPI and of bufalin on E/A was of borderline significance (Fig. 2D – G). Hemodynamic pressure–volume measurements were performed to assess left ventricular (LV) compliance. Importantly, treatment with bufalin or lycorine completely counteracted development of passive stiffness of the LV caused by the Ang II-infusion, evidenced by a reduction of the end-diastolic pressure–volume relationship (EDPVR) (Fig. 2I). In line with the heart weight data (Fig. 2B), cardiac mass
measured by echocardiography was slightly increased, but unaffected by bufalin or lycorine in the Ang II-treated groups (Fig. 2J).

Bufalin- and lycorine were detectable in cardiac tissue (and plasma) of mice, confirming their uptake into the heart upon systemic delivery (Supplemental Fig. 2D - E). Moreover, continuous monitoring of systolic and diastolic blood pressures in mice via telemetry showed that neither bufalin nor lycorine had any regulative impact on hypertension-induced by Ang II, excluding the possibility that bufalin or lycorine confer cardio-protection by reducing elevated blood pressure (Supplemental Fig. 2F - G). Of note, Ang II-infusion for eight weeks did not increase the number of terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) stained cells in the myocardium, ruling out the possibility that bufalin or lycorine exert cardio-protective effects by diminishing cardiomyocyte death (Supplemental Fig. 2H). In line with this, BAX/BCL2 ratio based on mRNA levels in the same cardiac tissues remained comparable within all groups tested (Supplemental Fig. 2I).

The anti-inflammatory effects of bufalin and lycorine have been previously described. In particular, attenuation of pro-inflammatory mediators such as TNFα and IL-1β could be demonstrated.19,21 Inflammation commonly precedes fibrosis-development in cardiac diseases. We therefore measured mRNA levels of TNFα and IL-1β in whole heart tissues. Although trends towards lower transcriptional activities of TNFα and IL-1β occurred upon bufalin treatment, Ang II-infusion did not lead to any significant activation of TNFα or IL-1β expression (Supplemental Fig. 2J - K).

Regarding the safety and tolerability of the compounds, no signs of kidney and liver damage by plasma markers were found (Supplemental Fig. 3A). This provides the first promising data for further development of the two substances bufalin and lycorine.
Bufalin and lycorine reverse fibrosis and recover cardiac function in angiotensin II-induced cardiac remodelling

We next aimed to test the beneficial effects of the identified anti-fibrotic compounds on cardiac fibrosis and function in a therapeutic setting; i.e. when cardiac fibrosis was already established. Therefore, treatment of Ang II-infused mice with bufalin or lycorine was started at a time point when cardiac fibrosis and diastolic dysfunction was already evident. Bufalin, lycorine or the solvent were injected intraperitoneally every other day for six consecutive weeks starting two weeks after start of the Ang II treatment (Fig. 3A). Treatment with both compounds significantly reduced overall fibrosis in the myocardium (Fig. 3B and Supplemental Fig. 3B) without affecting cardiomyocyte size (Supplemental Fig. 3C - D). The strong anti-fibrotic effect of both compounds is evidenced by detailed analysis of the perivascular and interstitial regions (Supplemental Fig. 4A - C).

Hemodynamic and echocardiography assessment revealed the progression of diastolic dysfunction to a more severe stage, evidenced by enlarged left atria, reduced dP/dt\textsubscript{min}, statistically significant increase of Tau and EDPVR, increased of E to A ratio, highly increased MPI and E to E’ peak ratio as well as a decreased deceleration time (Fig. 3D-H, Supplemental 4D, E, Table 1). Both compounds were able to partly reverse diastolic dysfunction; bufalin recovered hemodynamic parameters (dP/dt\textsubscript{min}, Tau and EDPVR) and both MPI and deceleration time (Fig. 3C-E, Supplemental Fig. 4E) and lycorine showed protective effects on diastolic dysfunction to a lesser degree with borderline significance (Fig. 3D - E). Both compounds reduced the elevated E to A and the LA area (Fig. 3F, Table 1) and tissue Doppler imaging confirmed faster tissue motion during diastole upon treatment with bufalin as represented by...
recovery of E to E’ ratios in Ang II-treated mice (Fig. 3G, Supplemental Fig. 4E, Table 1).

Moreover, 2-dimensional speckle-tracking strain echocardiography uncovered a significant reversal of the downshift of both radial and longitudinal strains (%) in the base, mid and apical regions of the heart triggered by Ang II-infusion upon treatment with bufalin and lycorine (Table 2, Supplemental Fig. 4F - H). Importantly, PV analysis revealed that bufalin and lycorine improved LV compliance, as evidenced by a significant reduction of EDPVR, a load independent parameter of LV stiffness (Fig. 3H), without affecting ESPVR (Supplemental Fig. 4D). Thus, systematic assessment of cardiac function unveiled a prominent reduction of passive stiffness of the LV upon systemic hypertension by the anti-fibrotic activity of the lead compounds (especially in the “therapeutic” set-up; e.g. a situation where treatment started only after fibrosis and diastolic dysfunction were already established). Ang II-infusion significantly increased LV mass in animals infused with the solvent only (controls), which remained unchanged in mice treated with bufalin or lycorine (Fig. 3I). Importantly, there was no difference between treatments within the control groups (bufalin or lycorine only versus vehicle only groups), indicating that the compounds are without cardiac effects under basal conditions (Fig. 3B-I).

To provide first insight into safety and tolerability also after the prolonged administration of the substances (six weeks), changes in the morphology, fibrosis, necrosis and inflammation of kidney and liver were evaluated (Supplemental Fig. 5A).

Interestingly, Ang II-infusion for eight weeks (Supplemental Fig. 5B) led to a less pronounced induction of fibrosis in female mice, which was however also ameliorated by the two lead compounds (Supplemental Fig. 5C). In line, MPI remained almost unchanged among groups tested (Supplemental Fig. 5D). In contrast, Ang II-infusion significantly increased
IVRT/AET in female mice infused with the solvent only (controls), which could be attenuated in animals treated with bufalin or lycorine (Supplemental Fig. 5E). The reduced responsiveness of female animals to Ang II-infusion as compared with age-matched male mice is likely due to the presence of estrogen in females, whose protective effects (hypertension and cardiovascular disease (CVD)) are well established.\textsuperscript{22,23}

Collectively, these data emphasize amelioration of cardiac function and fibrosis in the diseased heart in a therapeutic setting. This may open up novel opportunities for the future treatment of patients with established cardiac fibrosis and diastolic dysfunction.

**Lycorine and bufalin prevent the development of cardiac fibrosis and diastolic dysfunction in hypertensive rats**

Hypertension is one of the major causes for the development of HF with diastolic dysfunction. These patients typically exhibit heterogeneous histories of conglomerate risk factors, such as diabetes mellitus, dyslipidemia, obesity, atrial fibrillation, ageing or renal dysfunction.\textsuperscript{24} The Dahl salt-sensitive rat fed with a high salt diet represents the most frequently used model of systemic hypertension-induced HF with diastolic dysfunction, developing common comorbidities such as dyslipidemia and renal failure.\textsuperscript{25} Moreover, the close reproduction of the human cardiac pathophysiology further strengthens clinical relevance and thus was used as a supplementary model to test the efficacy of both anti-fibrotic compounds (Fig. 4A). Remarkably, evaluation of the hearts revealed that fibrosis-development was prevented by both bufalin and lycorine in rats fed with high salt diet. This was evident by near-normal values of collagen volume fraction (CVF), collagen cross-linking (CCL) as well as collagen deposition in whole heart sections of treated rats (Fig. 4B - D). Both bufalin and lycorine showed no significant impact on cardiomyocyte size in the Dahl salt-sensitive rat model (Fig. 4E). Collectively, these
results imply beneficial fibroblast-specific actions of bufalin and lycorine. As expected, echocardiographic evaluation of cardiac function revealed progressive diastolic dysfunction in rats fed with high salt diet, evidenced by the reversed hemodynamic parameters Tau, dP/dt_{min} (borderline significance) and increase of the E to A ratio. Both compounds improved diastolic properties, as reflected by significant reductions of MPI, IVRT/AET, the E to A and E to E’ peak ratios compared to control rats fed with high salt diet (Fig. 4F–I, Table 3, Supplemental Fig. 6A).

Importantly, treatment with both compounds had no impact on renal inflammation, tubular injury, loss of brush border, protein- and detritus-containing casts, thrombotic microangiopathy, glomerulosclerosis, nor arterio- or arteriolosclerosis induced in kidneys by the high salt diet in hypertensive rats (Supplemental Fig. 6B). Likewise, livers of the hypertensive rats showed loss of glycogen and steatosis, independently of compound treatment. In addition, vessel wall necrosis and thrombotic microangiopathy was only evident in hypertensive animals that did not receive lycorine (Supplemental Fig. 6C). Again, both compounds showed no effect on heart weight or lung weights (Supplemental Fig. 6D, E). Lung water content also has not been changed among groups (data not shown). Weekly dietary food intake was not affected by lead compounds injections (Supplemental Fig. 6F), excluding the possibility that the protective effects are being influenced by factors related to high salt diet consumption.

**Pro-fibrotic miR-671-5p is a common effector of the identified anti-fibrotic natural derived substances**

To elucidate a potential influence of the identified natural substances on endogenous miRNAs, key post-transcriptional regulators of gene expression, miRNA deep-sequencing in primary HCFs was performed. Interestingly, levels of the highly conserved miR-671-5p were consistently
and significantly reduced after treatment of primary HCFs with all 5 anti-fibrotic lead compounds from the initial screen in vitro (Fig. 5A - B). This result suggested that miR-671-5p might play a key-role in the fibrotic response. Indeed, miR-671-5p regulated both fibrosis and inflammatory pathways in HCFs. Specific overexpression of miR-671-5 in HCFs led to transcriptional activation of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), connective tissue growth factor (CTGF) as well as the pro-inflammatory cytokines interleukin 6 and 8 (IL-6 and IL-8), indicating that miR-671-5p is sufficient to induce fibrotic and inflammatory responses in vitro (Fig. 5C, Supplemental Fig. 7A). To delineate the mechanistic involvement of miR-671-5p in a cellular anti-fibrotic pathway activated by bufalin, impact of miR-671-5p overexpression on transcriptional activation of \( \alpha \)-SMA of primary HCFs upon treatment with bufalin was monitored. Bufalin treatment decreased \( \alpha \)-SMA expression in primary HCFs transfected with miR-mimic control or miR-671-5p, compared to DMSO control. Anti-fibrotic effects of bufalin on \( \alpha \)-SMA expression of primary HCFs is significantly disturbed upon overexpression of miR-671-5p, reflected by elevated \( \alpha \)-SMA expression (Fig. 5D), suggesting a role of miR-671-5p in the anti-fibrotic efficacy of bufalin.

Due to an intronic localization of miR-671-5p, expression of its host gene Chondroitin Polymerizing Factor (CHPF) was additionally determined in primary HCFs after treatment with the lead compounds. Expression levels of CHPF however did not change consistently in primary HCFs upon treatment with the identified molecules (Supplemental Fig. 7B) suggesting that the substances do not directly drive transcription of the miR-671-5p host gene CHPF. Consistently, increased miR-671-5p expression could be observed in fibrotic conditions in vivo. Cardiac stress was induced in wild-type mice via infusion of angiotensin II. Indeed, cell fractionation experiments using hearts of mice after implantation of minipumps filled with Ang
II or vehicle, showed a significant and fibroblast-specific activation of miR-671-5p (Fig. 5E). This activation was found to be counteracted by bufalin, confirming a possible functional importance of this miRNA in the anti-fibrotic action of bufalin also in vivo (Supplemental Fig. 7C).

In summary, highly conserved miR-671-5p (according to miRBase http://www.mirbase.org) was determined as a common effector of the lead compounds and as a key player of the fibrotic response in HCFs in vitro. Moreover, our studies underscore a functional role of miR-671-5p in fibrosis-development also in vivo and highlight its potential clinical importance.

**Anti-fibrotic selenoprotein P1 is a target of miR-671-5p in cardiac fibroblasts**

Bioinformatic analysis using TargetScanHuman (Version 7.1) predicted a 8mer miR-671-5p-binding site in the 3’ untranslated region (3’ UTR) of selenoprotein P1 (SEPP1) that is conserved also in the murine species (Fig. 6A). The cardio-protective role of the dietary trace element selenium (Se) has been shown in a plethora of cardiac stress conditions ranging from myocardial hypertrophy, diabetes- and metabolic syndrome-induced cardiac stress, ischaemia-reperfusion, homocysteine dysregulation to doxorubicin toxicity and, importantly, is dependent on its incorporation into selenoproteins. In line, effective silencing of SEPP1 in HCFs via siRNA chemistry led to enhanced COL1A1 expression in HCFs (Supplemental Fig. 7E and F).

Repression of SEPP1 by miRNA-671-5p would therefore support the detrimental activity of this miRNA. To validate the bioinformatic prediction via TargetScanHuman, we cloned the 3’ UTR of SEPP1 downstream of the firefly luciferase gene and found the normalized luciferase activity to be markedly reduced upon co-transfection of the construct with miR-671-5p mimic as compared to the miR-mimic control (Fig. 6B). To proof that SEPP1 is a target of miR-671-5p in
primary HCFs; *SEPP1*-levels upon overexpression of miR-671-5p were monitored. Levels of *SEPP1* were prominently and significantly decreased in primary HCFs after overexpression of miR-671-5p (Fig. 6C). These data validate *SEPP1* as target of miR-671-5p in primary HCFs. Importantly, *SEPP1* levels were found to be increased (whereas miR-671-5p levels were decreased) in primary HCFs following treatment with the lead anti-fibrotic substances, particularly Geldanamycin and Bufalin (Fig. 6D). These results suggest a protective role of *SEPP1*, possibly preventing myocardial stiffness also *in vivo*. Consistently, we found expression levels of *SEPP1* decreased in fibrotic cardiac tissue of mice infused with Ang II for 8 weeks. Importantly, Ang II-induced decline of *SEPP1* mRNA was effectively recovered by treatment with the lead compounds over a time-period of 6 weeks. However, no effect on *SEPP1* mRNA expression was observed by treatment with the compounds bufalin or lycorine alone (Fig. 6E). In line, rescued SEPP1 protein expression reduction by Ang II infusion has been shown in bufalin treated group compared to DMSO treated group (Supplemental Fig. 7H). Likewise, this normalization was also evident *in vitro*: bufalin significantly reversed the decline of SEPP1 protein associated with Ang II-stimulation of HCFs (Supplemental Fig. 7G). These results might explain the superior efficacy of bufalin both *in vitro* and in the therapeutic setting at applied doses (Fig. 1 and 3). To further establish the mechanistic role of *SEPP1* as downstream target of miR-671-5p, transcriptional activation of *α-SMA* in primary HCFs was followed after siRNA mediated silencing of *SEPP1* upon treatment with bufalin. The bufalin-induced decline of *α-SMA* expression in primary HCFs was restored by specific silencing of *SEPP1* in primary HCFs via siRNA chemistry (Fig. 6F), thereby replicating the impact of repressing *SEPP1* by miR-671-5p overexpression (Fig. 5D). Collectively, these results suggest that *SEPP1* might be
the anti-fibrotic target of miR-671-5p that partly explains the mechanistic involvement of this miRNA in the anti-fibrotic efficacy of bufalin (Fig. 7).

Discussion

Here we show the effective anti-fibrotic potential of the two natural-derived compounds bufalin and lycorine both in primary HCFs and in multiple rodent models of cardiac remodelling in preventive as well as therapeutic settings. Particularly bufalin preserved global and diastolic function of the heart, correlating with a significant reduction of collagen deposition. Moreover, especially bufalin ameliorated cardiac function when treatment was started at a time point of already established diastolic dysfunction, underscoring its clinical translational importance. Both substances were effectively taken up by the heart upon systemic delivery in vivo. Continuous monitoring of blood pressure via telemetry uncovered that neither bufalin nor lycorine show any antihypertensive action upon Ang II infusion, excluding the possibility that bufalin or lycorine confer cardio protection by reducing elevated blood pressure. Strikingly, when employing the clinically relevant Dahl salt-sensitive rat model, fibrosis and diastolic dysfunction were effectively abolished by bufalin and lycorine. This result highlights the reproducibility of the anti-fibrotic effects seen in hypertensive mice in an additional species and model.

The cardio protective potential of bufalin and lycorine may be attributed to their suppressive effects on proliferation and collagen production of cardiac fibroblasts, the effector cells of fibrosis in the diseased heart. Mechanistically, we identified miR-671-5p as a common key player of the lead candidates mediating fibrosis in primary HCFs. Hence, modulation of miR-671-5p in the heart might be harnessed as an alternative therapeutic strategy for treatment of cardiac fibrotic diseases. However, careful risk analysis would be required since miR-671-5p is
ubiquitously expressed in various cell types and organs (Supplemental Fig. 7D) which may give rise to non-intended off-target effects. Additionally, no changes have been shown in fibrosis and inflammatory markers with sole inhibition of miR-671-5p (Supplemental Fig. 7A), whereas significant reductions of miR-671-5p in natural compound treated animals (Fig. 5C), suggesting presence of additional mechanisms to be involved in the observed anti-fibrotic effect of the compounds.

Interestingly, we found that pro-fibrotic effects of miR-671-5p might be mediated by repression of its target SEPP1. This protein transports dietary trace element Selenium from the liver to target tissues and was described as part of the antioxidant defence line and important cardio-protector. Here we show that SEPP1 is also a target of miR-671-5p in primary HCFs, which express this protein in remarkably high amounts. Our findings imply an anti-fibrotic, protective role of SEPP1, possibly preventing myocardial stiffness also in vivo. Consistently, we found expression levels of SEPP1 decreased in fibrotic cardiac tissue of our murine model of diastolic dysfunction caused by prolonged Ang II-infusion (therapeutic approach). Importantly, especially bufalin significantly recovered SEPP1 levels, possibly explaining apparent higher effectivity of this compound in a therapeutic setting.

Despite some efforts to develop new therapeutic approaches for the treatment of patients with HF with diastolic dysfunction and its progression to end-stage systolic HF, life-expectancy of patients remains unacceptably low. In line, cardiovascular diseases still account for the highest number of deaths worldwide. Myocardial fibrosis is a hallmark of cardiac remodeling and a major determinant for both development and progression of HF. Thus, a breakthrough discovery for a therapeutic strategy to primarily target maladaptive responses in cardiac fibroblasts would be indispensable. We here provide evidence for cardio protective, anti-fibrotic
and promising safety-tolerability profile of the natural compounds bufalin and lycorine, hereby setting the ground for prospective preclinical and clinical studies for both prevention and therapy of fibrotic cardiac diseases. To date, no promising treatment for patients with diastolic dysfunction exists. Indeed, an understanding of the association between diastolic dysfunction and clinical syndrome of HF is still poor. Therefore, future clinical testing of compounds that show strong cardiac anti-fibrotic effects and improve diastolic function in selected HF patients should be explored.

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Disclosures

KS, QTD and TT have filed a patent about the use of anti-fibrotic natural compounds. JF and TT have filed patents about the use of non-coding RNAs in cardiovascular diseases. TT has licensed patents about the use of noncoding RNAs. TT and SB are founders and hold shares of Cardior Pharmaceuticals GmbH.

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Jessica Schmitz, MSc8; Christina Brandenberger, PhD9; Dominik N. Müller, PhD10;
Nicola Wilck, MD10,11; Volkhard Kaever, PhD12; Heike Bähre, PhD12;
Sandor Batkai, MD, PhD1; Jan Fiedler, PhD1; Kevin M. Alexander, MD2;
Bradley M. Wertheim, MD13; Sudeshna Fisch, PhD5; Ronglih Liao, PhD2,5;
Javier Diez, MD, PhD3,4,14; Arantxa González, PhD3,4 and Thomas Thum, MD, PhD1,15

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References

fibrosis with diagnostic and/or therapeutic potential. Eur J Heart Fail. 2015;17:764-771.
doi.org/10.1002/ejhf.312
doi.org/10.1016/j.toxlet.2013.01.018
17. CHANG YW, ZHAO YF, CAO YL, GU W, PANG J, ZHAN HS. Bufalin exerts inhibitory effects on IL-1beta-mediated proliferation and induces apoptosis in human rheumatoid arthritis.


**Table 1.** Echocardiographic and hemodynamic parameters of therapeutic approach in Ang II-infused mouse model.

<table>
<thead>
<tr>
<th>Millar parameters</th>
<th>Vehicle group</th>
<th>Angiotensin II group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (ms)</td>
<td>DMSO</td>
<td>Bufalin</td>
</tr>
<tr>
<td></td>
<td>453.8±36.07</td>
<td>437.3±20.94</td>
</tr>
<tr>
<td></td>
<td>0.087±0.05</td>
<td>0.090±0.03</td>
</tr>
<tr>
<td>EDPVR</td>
<td>2.01±0.60</td>
<td>3.89±2.11</td>
</tr>
<tr>
<td>Ees/Ea</td>
<td>1.19±0.36</td>
<td>1.99±1.22</td>
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<tr>
<td>dP/dt max (mmHg/s)</td>
<td>6175±1498</td>
<td>5674±1608</td>
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<tr>
<td>dP/dt min (mmHg/s)</td>
<td>-6910±1618</td>
<td>-6174±1770</td>
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<tr>
<td>Tau (ms)</td>
<td>6.25±0.71</td>
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<tr>
<td>SV (mL)</td>
<td>41.46±4.86</td>
<td>36.03±7.31</td>
</tr>
<tr>
<td>EF (%)</td>
<td>57.12±16.28</td>
<td>57.55±17.67</td>
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</tbody>
</table>

Data are expressed as mean±SD. LVEDV, Left Ventricle End Diastolic Volume; LVESV, Left Ventricle End Systolic Volume; EF, Ejection Fraction; EDD, End Diastolic Diameter; ESV, End Systolic Diameter; FS, Fractional Shortening; E, Early mitral inflow diastole peak; A, Late mitral inflow diastole peak; E’, Early annular diastole peak; A’, Late annular diastole peak; IVRT, Isovolumetric Relaxation Time; IVCT, Isovolumetric Contraction Time; LVET, Left Ventricular Ejection Time; LA:d, Left Atrial diastolic size; LA:s, Left Atrial systolic size, EDPVR, End Diastolic Pressure Volume Relationship; ESPVR, End Systolic Pressure Volume Relationship; Ea, Arterial Elasticance; dP/dtmax, point of maximum pressure increase; dP/dtmin, point of maximum pressure decrease; Tau (Weiss), time constant of active relaxation; SV, Stroke Volume, two-way ANOVA, Tukey’s multiple comparisons test, * refers to comparison between vehicle-treated and AngII-treated mice (* P<0.05) and # refers to effect of compounds in AngII-treated mice (# P<0.05).
Table 2. Global strain analysis of cardiac 6 segments of therapeutic approach in Ang II-infused mouse model.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
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<tr>
<td></td>
<td>RS (%)</td>
<td></td>
<td>PL (%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RS (%)</td>
<td>DMSO</td>
<td>Bufalin</td>
<td>Lycorine</td>
<td>DMSO</td>
<td>Bufalin</td>
<td>Lycorine</td>
<td>DMSO</td>
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<tr>
<td>Seg 2</td>
<td>48.49±11.89</td>
<td>35.28±12.58</td>
<td>31.67±9.10</td>
<td>23.87±7.37</td>
<td>28.66±12.13</td>
<td>34.13±14.57</td>
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<tr>
<td>Seg 4</td>
<td>41.42±17.66</td>
<td>50.60±21.74</td>
<td>35.38±7.53</td>
<td>31.41±14.77</td>
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<td>Seg 5</td>
<td>48.76±19.91</td>
<td>49.27±20.17</td>
<td>44.20±6.682</td>
<td>32.19±10.67</td>
<td>31.43±11.20</td>
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<tr>
<td>Seg 6</td>
<td>34.53±17.60</td>
<td>32.51±23.02</td>
<td>27.32±9.81</td>
<td>20.19±9.14</td>
<td>30.73±13.94</td>
<td>30.37±11.98</td>
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</table>

Data are expressed as mean±SD. RS, Radial strain; LS, Longitudinal strain; Seg 1, Posterior base; Seg 2, Posterior mid; Seg 3, Posterior apex; Seg 4, Anterior base; Seg 5, Anterior mid; Seg 6, Anterior apex
Table 3. Echocardiographic and hemodynamic parameters of the therapeutic study done in the high salt diet induced rat model.

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<tr>
<th>Echo parameters</th>
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<td></td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td>Bufalin</td>
<td>Lycorine</td>
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<tr>
<td>Heart rate (ms)</td>
<td>395.4±26.46</td>
<td>368.7±40.85</td>
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<tr>
<td></td>
<td>369.6±56.78</td>
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<tr>
<td>LVEDV (mL)</td>
<td>299.6±26.46</td>
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<td>299.6±36.14</td>
<td>320.5±45.95</td>
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<tr>
<td>LVESV (mL)</td>
<td>44.88±11.33</td>
<td>80.33±28.97*</td>
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<td></td>
<td>89.19±37.43</td>
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<tr>
<td>EF (%)</td>
<td>83.15±5.04</td>
<td>72.36±6.14*</td>
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<tr>
<td></td>
<td>70.38±12.20</td>
<td>76.07±3.57</td>
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<tr>
<td>EDD (mm)</td>
<td>7.16±0.39</td>
<td>7.36±0.68</td>
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<td>7.50±0.56</td>
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<td>ESD (mm)</td>
<td>3.30±0.349</td>
<td>4.19±0.61*</td>
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<td>4.34±0.83</td>
<td>4.14±0.33</td>
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<td>FS (%)</td>
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<td>43.07±5.41*</td>
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<td></td>
<td>42.15±10.28</td>
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<tr>
<td>Peak E (cm/sec)</td>
<td>846±151.6</td>
<td>870.5±113.4</td>
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<td>904.1±241.2</td>
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<tr>
<td>Peak A (cm/sec)</td>
<td>603.8±96.89</td>
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<td>640.7±181.5</td>
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<td>E/A ratio</td>
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<td>1.43±0.18*</td>
<td>1.31±0.23*</td>
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<td>E’ (cm/sec)</td>
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<td>-49.85±26.89</td>
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<td>A' (cm/sec)</td>
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<td>-46.46±12.81</td>
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<td>IVRT (msec)</td>
<td>11.56±2.69</td>
<td>20.74±3.60*</td>
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<td>17.52±3.26(0.09)</td>
<td>13.93±2.22*</td>
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<tr>
<td>IVCT (msec)</td>
<td>16.3±3.66</td>
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<td>18.29±1.91(0.06)</td>
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<td>LVET (msec)</td>
<td>61.95±4.38</td>
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<td>66.23±3.58(0.08)</td>
<td>68.23±7.30(0.09)</td>
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<table>
<thead>
<tr>
<th>Millar parameters</th>
<th>Normal diet</th>
<th>High salt diet</th>
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<tbody>
<tr>
<td></td>
<td>DMSO</td>
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</tr>
<tr>
<td></td>
<td>Bufalin</td>
<td>Lycorine</td>
</tr>
<tr>
<td>Heart rate (ms)</td>
<td>346.1±21.65</td>
<td>349.7±22.12</td>
</tr>
<tr>
<td></td>
<td>345.1±20.47</td>
<td>362±37.51</td>
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<tr>
<td>EDPVR (mmHg/uL)</td>
<td>0.054±0.036</td>
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<tr>
<td></td>
<td>0.048±0.021</td>
<td>0.055±0.032</td>
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<tr>
<td>ESPVR (mmHg/uL)</td>
<td>0.89±0.07</td>
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<tr>
<td></td>
<td>0.91±0.47</td>
<td>1.03±0.52</td>
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<tr>
<td>Ees/Ea</td>
<td>1.77±0.21</td>
<td>2.40±1.03</td>
</tr>
<tr>
<td></td>
<td>1.39±0.85</td>
<td>1.73±1.06</td>
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<tr>
<td>dP/dT max (mmHg/s)</td>
<td>6890±1203</td>
<td>5912±537.9(0.07)</td>
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<td>6130±647.3</td>
<td>7510±951.4*</td>
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<td>dP/dT min (mmHg/s)</td>
<td>-8965±1674</td>
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<td>-8426±1164</td>
<td>-9613±2616(0.10)</td>
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<tr>
<td>Tau (ms)</td>
<td>8.90±1.03</td>
<td>10.06±0.98(0.06)</td>
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<tr>
<td></td>
<td>9.96±1.29</td>
<td>9.20±0.76(0.07)</td>
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<tr>
<td>SV (mL)</td>
<td>228.5±46.97</td>
<td>176±33.52(0.09)</td>
</tr>
<tr>
<td></td>
<td>186.1±35.28</td>
<td>216±50.28</td>
</tr>
<tr>
<td>EF (%)</td>
<td>92.92±8.46</td>
<td>73.29±10.58*</td>
</tr>
<tr>
<td></td>
<td>74.17±13.43</td>
<td>82.97±14.85</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. EDPVR, End Diastolic Pressure Volume Relationship; ESPVR, End Systolic Pressure Volume Relationship; Ea, Arterial Elastance; dP/dTmax, point of maximum pressure increase; dP/dTmin, point of maximum pressure decrease; Tau, time constant of active relaxation; SV, Stroke Volume; EF, Ejection Fraction; LVEDV, Left Ventricle End Diastolic Volume; LVESV, Left Ventricle End Systolic Volume; EDD, End Diastolic Diameter; ESD, End Systolic Diameter; FS, Fractional Shortening; E, Early mitral inflow diastole peak; A, Late mitral inflow diastole peak; E’, Early annular diastole peak; A’, Late annular diastole peak; IVRT, Isovolumic Relaxation Time; IVCT, Isovolumic Contraction Time; LVET, Left Ventricular Ejection Time; LA;d, Left Atrial diastolic size; LA;s, Left Atrial systolic size, two-way ANOVA, Tukey’s multiple comparisons test, * refers to comparison DMSO control between normal-diet and high salt-diet rat (* P<0.05) and # refers to effect of compounds in high salt-diet rat (# P<0.05).
Figure Legends

Figure 1. Bufalin and lycorine potently and specifically repress fibrotic responses in human cardiac fibroblasts. (A) Filtering strategy, followed by *in vitro* and *in vivo* pipeline of analysis, uncovers anti-fibrotic lead compounds bufalin and lycorine. (B) Functional screen of 480 natural-derived substances *in vitro* yielding natural compounds inhibiting proliferation of HCFs. The cells were incubated for 24h as indicated and proliferation of HCFs was measured by BrdU-ELISA. 15 candidates inhibiting fibroblast proliferation FC ≥ 75% are highlighted by red rectangle. (C) Dose-dependent inhibitory effects of bufalin, gitoxigenin, lycorin, anisomycin and geldanamycin on proliferation of HCFs. The cells were incubated for 24h as indicated and proliferation of HCFs was measured by BrdU-ELISA (one-way ANOVA, Dunnett’s multiple comparisons test, n=4-6). (D) Positively validated hits do not induce cell death in HCFs. Cells were treated with respective compounds for 24h as indicated and subjected to FACS analysis after Annexin-7AAD-staining (unpaired t-test, n=3). (E) Dose-dependent inhibitory effects of bufalin and lycorine on proliferation of HCFs are fibroblast-specific as evidenced by no impact of the same concentrations of respective substances on proliferation of the cardiomyocyte cell line HL-1. The cells were treated with the compounds for 24h as indicated and proliferation of and HL-1 cells was measured by BrdU-ELISA (one-way ANOVA, Dunnett’s multiple comparisons test, n=3). (F) Bufalin and to a lesser extend also lycorine decrease expression levels of COL1A1 in HCFs shown in a representative Western Blot. Cells were treated with respective substances for 24h as indicated, lysed and analysed for COL1A1 protein levels (normalised to GAPDH) (unpaired t-test).

All values from (C) – (F) are represented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001.
# p-value = 0.265

COL1A1 – alpha-1 type I collagen; DMSO = dimethylsulfoxid; FC = fold change; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; ns = not significant

**Figure 2. Bufalin and lycorine protect from angiotensin II-induced fibrotic cardiac disease in mice.** (A) Schematic representation of the preventive *in vivo* study using a murine model of hypertensive heart disease. Diastolic dysfunction was induced in C57BL/6 mice via implantation of angiotensin II filled minipumps and Bufalin or Lycorine was injected intraperitoneally every other day during two consecutive weeks starting one day after the operation. Fourteen days after the operation hearts were explanted for biochemical and histological analysis. (B) Reduced heart-to-body weight ratios (HW/BW) upon treatment as indicated compared to control animals (one-way ANOVA, Tukey’s multiple comparisons test, n = 14/17/17/14). (C) Prevention of collagen deposition shown in representative images of cardiac histological sections of the whole heart and the quantification of Picro-sirius red-stained areas upon administration of bufalin and lycorine (one-way ANOVA, Tukey’s multiple comparisons test, n = 10/11/10/11), scale bar 1mm. (D) IVCT, IVRT, AET, E and A waves are measured by pulsed wave (PW) Doppler from the apical 4 chamber view from the lateral mitral valve (top). Early (E’) and atrial (A’) peak velocities are measured from Tissue Doppler signal at the mitral annulus (bottom). Preserved Myocardial Performance Index (MPI) (E; one-way ANOVA, Tukey’s multiple comparison, n = 11/21/16/12), deceleration time (DT) (F; one-way ANOVA, Tukey’s multiple comparisons test, n = 11/21/16/12), and increased E to A peak ratio (G; one-way ANOVA, Tukey’s multiple comparisons test, n = 8/16/11/7), improved E’ peak value (H; one-way ANOVA, Tukey’s multiple comparisons test, n = 8/16/11/7) after treatment with compounds, as indicated. (I)
Preservation of left ventricular (LV) compliance by bufalin or lycorine, assessed by end-diastolic pressure–volume relationship (EDPVR) obtained by linear fits of EDPVR slope, due to the shift of PV-loops after transient vena cava occlusions (one-way ANOVA, Tukey’s multiple comparisons test, n = 8/9/6/8). (J) Significant increase in left ventricular mass (LV mass) by AngII-infusion in animals treated with the solvent only (controls), but not in mice treated with bufalin or lycorine (one-way ANOVA, Tukey’s multiple comparisons test, n = 8/11/9/9).

All values from (B) – (J) are represented as mean±SEM. *p<0.05; **p<0.01.

d = day; HW/BW = heart weight to body weight ratio, MPI = Myocardial Performance Index; IVRT = isovolumetric relaxation time; AET= aortic ejection time; E = early mitral flow velocity; A = late mitral flow velocity; E’ = early tissue doppler velocity; A’ = late tissue doppler velocity; EDPVR = end-diastolic pressure–volume relationship; LV = left ventricular; other abbreviations as in Fig. 1

Figure 3. Bufalin and lycorine reverse established fibrosis and improve heart function in hypertensive mice. (A) Therapeutic in vivo study using a murine model of systemic hypertension. Bufalin, lycorine or DMSO were injected intraperitoneally every other day for six consecutive weeks starting two weeks after the implantation of minipumps filled with angiotensin II. (B) Significant amelioration of cardiac fibrosis in established diastolic heart failure upon treatment with bufalin and lycorine by 50 % (two-way ANOVA, Tukey’s multiple comparisons test, n = 19/8/7/17/18/16). (C) Representative images of hemodynamic PV loops. Both compounds were able to partly reverse diastolic dysfunction; bufalin recovered hemodynamic parameters (dP/dtmin, Tau and EDPVR) compared to Angiotensin II group. (D - F) Strong trend of reduction of MPI (one-way ANOVA, n = 19/8/7/17/18/16) and prolongation
of DT (two-way ANOVA, Tukey’s multiple comparisons test, n = 19/8/7/17/18/16) by the lead compounds as well as decreased E to A peak ratio (two-way ANOVA, Tukey’s multiple comparisons test, n = 18/8/7/14/15/15) upon treatment with compounds. (G) Strong trend in reduction of E to E’ peak ratio upon treatment with both bufalin and lycorine. (two-way ANOVA, Tukey’s multiple comparisons test, n = 14/8/7/14/14/15). (H) Significant improvement of load-independent EDPVR by bufalin and lycorine upon systemic hypertension (two-way ANOVA, Tukey’s multiple comparisons test, n = 14/8/7/14/14/15). (I) Significant increase in LV mass by Ang II-infusion in animals treated with the solvent only (controls), but not in mice treated with bufalin or lycorine (two-way ANOVA, Tukey’s multiple comparisons test, n = 19/8/7/16/17/16).

All values from (B) – (I) are represented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Abbreviations as in Fig. 1 – 2.

**Figure 4. Lycorine and bufalin diminish fibrosis, diastolic dysfunction and pulmonary congestion in the Dahls salt-sensitive rat.** (A) In vivo study using a model of hypertension induced diastolic dysfunction. Lycorine, bufalin or DMSO was injected intraperitoneally every other day for five consecutive weeks starting two weeks after induction of hypertension by high salt diet. (B - D) Effective and significant reduction of collagen volume fraction (CVF) (one-way ANOVA, Tukey’s multiple comparisons test, n = 17/16/10/13), collagen cross-linking (CCL) (one-way ANOVA, Tukey’s multiple comparisons test, n = 17/16/10/13) and collagen accumulation in the myocardium as shown in representative images of picrosirius red staining; scale bar 1mm. (E) No significant impact on cardiomyocyte area shown in representative images and corresponding quantification of heart sections (one-way ANOVA, Tukey’s multiple
comparisons test, n = 17/16/10/13); scale bar: 200 μm. (F - H) Significantly ameliorated MPI and IVRT/AET in compounds treated rats compared to high salt diet rats (one-way ANOVA, Tukey’s multiple comparisons test, n = 10/9/10/13) as well as significant reduction of E to A peak ratio (one-way ANOVA, Tukey’s multiple comparisons test, n = 10/9/10/13) upon treatment with lycorine, not with bufalin. (I) Representative PW Doppler and Tissue Doppler images from each group.

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

CVF = collagen volume fraction; CCL = collagen cross-linking; other abbreviations as in Fig. 1 – 2

Figure 5. Anti-fibrotic substances converge on pro-fibrotic miR-671-5p. (A) Representative heatmap of differentially regulated miRNAs (unpaired t-test p-value <0.05) and (B) validation of significantly reduced miR-671-5p expression in primary HCFs upon treatment as indicated compared to control (unpaired t-test, n=3). (C) Activation of fibrosis markers α-SMA, CTGF and pro-inflammatory cytokines IL-6 and IL-8 in primary HCFs after overexpression of miR-671-5p (unpaired t-test, n=5, miR-mimic control vs miR671-5p mimic). (D) Restoration of diminished α-SMA expression of primary HCFs after treatment with bufalin by miR-671-5p (two-way ANOVA, Tukey’s multiple comparisons test, DMSO control vs 1μM Bufalin; unpaired t-test, miR-mimic control vs miR-671-5p mimic; n=5). (E) MiR-671-5p expression in murine heart cell fractions after infusion with angiotensin II for 2 weeks (unpaired t-test, n = 6/10). All values from (B) – (E) are represented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
α-SMA = α-smooth muscle actin; CTGF = connective tissue growth factor; IL-6 = interleukin 6; IL-8 = interleukin 8; RNU48 = small-nucleolar RNA48; 18S rRNA = 18S ribosomal RNA; other abbreviations as in Fig. 1.

Figure 6. miR-671-5p drives fibrosis via repression of selenoprotein P1. (A) Predicted base-pairing between the seed sequence of human miR-671-5p and target sequence in the 3’ untranslated (3’ UTR) region of selenoprotein P1 (SEPP1) (TargetScanHuman, version 7.1). (B) Luciferase activity levels upon transfection of a luciferase construct containing the 3’UTR of SEPP1 with miR-mimic control or miR-671-5p mimic (one sample t-test, n=27 replicates). (C) Significant reduction of SEPP1 mRNA levels in primary HCFs after overexpression of miR-671-5p (unpaired t-test, n=3). (D) SEPP1 levels follow the opposite pattern as compared to miR-671-5p in primary HCFs upon treatment with the lead anti-fibrotic substances (unpaired t-test, n=2-3). (E) Decrease of SEPP1 levels in cardiac tissue after 8 weeks Ang II-infusion (therapeutic mouse study) is significantly counteracted by bufalin and lycorine (two-way ANOVA, Tukey’s multiple comparisons test, n = 18/8/7/17/18/16). (F) Restoration of diminished α-SMA expression in primary HCFs after treatment with bufalin by siRNA-mediated silencing of SEPP1 (two-way ANOVA, Sidak’s multiple comparisons test, DMSO control vs 1μM Bufalin; unpaired t-test, siRNA control vs siRNA SEPP1; n=4).

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

SEPP1 = selenoprotein P1; other abbreviations as in Fig. 1.

Figure 7. Mechanistic overview. Proposed therapeutic mode of action (MOA) for the anti-fibrotic compound bufalin and lycorine in angiotensin II-mediated diastolic dysfunction.
Treatment with the lead natural compound results in a decline in miR-671-5p levels in cardiac fibroblasts, which in turn, leads to de-repression of its target \textit{SEPP1}. 
**A**

Library of 480 natural compounds selected for a maximum of chemical diversity

Bufalin
Gitoxigenin
Geldanamycin
Lycorine
Anisomycin

**In vivo model**
Dahl salt sensitive rat

Confirmation of cardioprotection in a second species

**In vitro model**
primary human cardiac fibroblasts

Proliferation-inhibition
Exclusion of cell death
Fibroblast specificity
Prevention of fibrosis
Toxicological safety
Reversal of fibrosis

Exclusion of Anisomycin

**In vivo model**
murine angiotensin II–induced cardiac fibrosis

Exclusion of Gitoxigenin
Geldanamycin

**B**

**C**

**D**

**E**

**F**

GAPDH

COL1A1/GAPDH

138 kDa

In vitro model
primary human cardiac fibroblasts

Positive cells [%]

GAPDH

COL1A1/GAPDH

138 kDa

In vivo model
murine angiotensin II–induced cardiac fibrosis

Exclusion of Gitoxigenin
Geldanamycin

Exclusion of Anisomycin

Positive cells [%]

Proliferation

FC of DMSO control

DMSO control

0.1 1 10

Lycorine (µM)

0.0

0.5

1.0

1.5

ns

***

Proliferation

FC of DMSO control

DMSO control

0.1 1 10

Anisomycin (µM)

0.0

0.5

1.0

1.5

ns

***

Proliferation

FC of DMSO control

DMSO control

0.1 1 10

Geldanamycin (µM)

0.0

0.5

1.0

1.5

ns

ns

***

Proliferation

FC of DMSO control

DMSO control

1 5 10

Bufalin (µM)

0.0

0.5

1.0

1.5

ns

ns

***

Proliferation

FC of DMSO control

DMSO control

1 5 10

Lycorine (µM)

0.0

0.5

1.0

1.5

ns

***

Proliferation

FC of DMSO control

DMSO control

1 µM Bufalin

DMSO control

5 µM Lycorine

DMSO control

1 µM Bufalin

DMSO control

5 µM Lycorine

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A. Implantation of angiotensin II (14 days)

B. Assessment of cardiac function and fibrosis

C. Fibrosis [%]

D. PW Doppler

E. Deceleration Time [ms]

F. MPI

G. E/A

H. E'

I. EDPVR

J. LV mass [mg]
Figure 3

A. I.p. injection of bufalin or lycorine

Implantation of angiotensin II (56 days)
Assessment of cardiac function and fibrosis

B. Fibrosis [%]

DMSO Bufalin Lycorine

DMSO Bufalin Lycorine

C. LV Pressure [mmHg]

Mean ESPVR: 2.01±0.60
Mean EDPVR: 0.087±0.05

Mean ESPVR: 3.89±2.11
Mean EDPVR: 0.090±0.03

Mean ESPVR: 1.84±0.66
Mean EDPVR: 0.066±0.03

Mean ESPVR: 3.08±1.5
Mean EDPVR: 0.26±0.09

Mean ESPVR: 2.91±1.48
Mean EDPVR: 0.087±0.03

Mean ESPVR: 4.35±2.12
Mean EDPVR: 0.084±0.08

D. Mean ESPVR: 2.01±0.60
Mean EDPVR: 0.087±0.05

E. Deceleration time [ms]

F. E/A

G. E/E'

H. EDPVR

I. LV mass
I.p. injection of bufalin or lycorine

**Figure 4**

A. L.p. injection of bufalin or lycorine

B. Graph showing the effect of DMSO control, bufalin, and lycorine on CVF and CCL.

C. Graph showing the effect of DMSO control, bufalin, and lycorine on cardiac function.

D. Micrographs showing cardiac tissue sections under different conditions.

E. Micrographs showing cardiac tissue sections under different conditions.

F. Graph showing the effect of DMSO control, bufalin, and lycorine on myocardial infarction size.

G. Graph showing the effect of DMSO control, bufalin, and lycorine on IVRT/AET.

H. Graph showing the effect of DMSO control, bufalin, and lycorine on E/A ratio.

I. PW Doppler and Tissue Doppler images under different conditions.
Figure 5
Figure 6

A

Predicted consequent pairing of target region (top) and miRNA (bottom)

Position 133-140 of SEPP1 3' UTR

5'...GAAUCUACUGAUAUUGCCUCCA...

3'...GAGGUCGGGAGGUUGCCGAGAA...

B

Luciferase activity

FC of miR-mimic control

DMSO control

1µM Gitoxigenin

miR-671-5p mimic

C

SEPP1/18S rRNA

FC of miR-mimic control

DMSO control

1µM Gitoxigenin

miR-671-5p mimic

D

SEPP1/18S rRNA

FC of miR-mimic control

DMSO control

1µM Anisomycin

E

SEPP1/18S rRNA

FC of DMSO control

DMSO control

1µM Geldanamycin

F

SEPP1/18S rRNA

FC of DMSO control

DMSO control

1µM Buffalin

Vehicle

AngiotensinII

siRNA control

siRNA SEPP1

α −SMA/18S rRNA

DMSO control

1µM Buffalin

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Circulation
Cardiac stress
LV overload

Cardiac fibrosis

Bufalin
(from Chinese toad venom)

miR-671-5P

Cardiac fibroblasts

Lycorine
(from Amaryllidaceae species)

SEPP1

other factors

Cardiac fibrosis / Passive stiffness

Diastolic function