

Original Articles Part 2

Angiotensin Type 2 Receptor Stimulation Ameliorates Left Ventricular Fibrosis and Dysfunction via Regulation of Tissue Inhibitor of Matrix Metalloproteinase 1/Matrix Metalloproteinase 9 Axis and Transforming Growth Factor β 1 in the Rat Heart

Dilyara Lauer, Svetlana Slavic, Manuela Sommerfeld, Christa Thöne-Reineke, Yuliya Sharkovska, Anders Hallberg, Bjorn Dahlöf, Ulrich Kintscher, Thomas Unger, Ulrike Muscha Steckelings,* Elena Kaschina*

Abstract—Left ventricular (LV) remodeling is the main reason for the development of progressive cardiac dysfunction after myocardial infarction (MI). This study investigated whether stimulation of the angiotensin type 2 receptor is able to ameliorate post-MI cardiac remodeling and what the underlying mechanisms may be. MI was induced in Wistar rats by permanent ligation of the left coronary artery. Treatment with the angiotensin type 2 receptor agonist compound 21 (0.03 mg/kg) was started 6 hours post-MI and continued for 6 weeks. Hemodynamic parameters were measured by echocardiography and intracardiac catheter. Effects on proteolysis were studied in heart tissue and primary cardiac fibroblasts. Compound 21 significantly improved systolic and diastolic functions, resulting in improved ejection fraction ($71.2 \pm 4.7\%$ versus $53.4 \pm 7.0\%$; $P < 0.001$), fractional shortening ($P < 0.05$), LV internal dimension in systole ($P < 0.05$), LV end-diastolic pressure (16.9 ± 1.2 versus 22.1 ± 1.4 mmHg; $P < 0.05$), ratio of early (E) to late (A) ventricular filling velocities, and maximum and minimum rate of LV pressure rise ($P < 0.05$). Compound 21 improved arterial stiffness parameters and reduced collagen content in peri-infarct myocardium. Tissue inhibitor of matrix metalloproteinase 1 was strongly upregulated, whereas matrix metalloproteinases 2 and 9 and transforming growth factor β 1 were diminished in LV of treated animals. In cardiac fibroblasts, compound 21 initially induced tissue inhibitor of matrix metalloproteinase 1 expression followed by attenuated matrix metalloproteinase 9 and transforming growth factor β 1 secretion. In conclusion, angiotensin type 2 receptor stimulation improves cardiac function and prevents cardiac remodeling in the late stage after MI, suggesting that angiotensin type 2 receptor agonists may be considered a future pharmacological approach for the improvement of post-MI cardiac dysfunction. (*Hypertension*. 2014;63:e60-e67.) • [Online Data Supplement](#)

Key Words: matrix metalloproteinase 9 ■ myocardial infarction ■ receptor, angiotensin, type 2 ■ TIMP1 protein, human

Heart failure (HF) secondary to myocardial infarction (MI) remains a major source of morbidity and mortality, despite significant advances in therapies developed for this disease.¹ The major determinant of survival after recovery from MI is left ventricular (LV) remodeling, which has been strongly associated with clinical outcomes in numerous HF trials.²

The cardiac renin–angiotensin–aldosterone system, which is highly activated after MI and in HF,³ has been implicated in

the process of vascular and cardiac remodeling. Angiotensin II through its type 1 receptor (AT₁R) induces myocardial hypertrophy, fibrosis, and cardiomyocyte apoptosis. Consequently, AT₁R antagonists inhibit fibrosis in post-MI hearts.⁴ Both angiotensin-converting enzyme inhibitors and AT₁R antagonists are cardioprotective with regard to post-MI LV dysfunction.^{4,5} Beneficial effects of AT₁R blockade have at least, in part, been attributed to stimulation of unopposed angiotensin

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From the Center for Cardiovascular Research and Institute of Pharmacology (D.L., S.S., M.S., C.T.-R., U.K., T.U., M.S., E.K.), Forschungseinrichtung für Experimentelle Medizin (C.T.-R.), and Institute of Vegetative Anatomy (Y.S.), Charité – Universitätsmedizin, Berlin, Germany; Department of Biomedical Sciences, Institute of Physiology, Pathophysiology and Biophysics, University of Veterinary Medicine, Vienna, Austria (S.S.); Department of Medicinal Chemistry, Uppsala University, Uppsala, Sweden (A.H.); Sahlgrenska University Hospital, Gothenburg, Sweden (B.D.); the Cardiovascular Research Institute Maastricht School for Cardiovascular Diseases, Maastricht University, Maastricht, The Netherlands (T.U.); and Department of Cardiovascular and Renal Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark (U.M.S.).

*These authors contributed equally to this work.

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Correspondence to Elena Kaschina, Center for Cardiovascular Research and Institute of Pharmacology, Charité – Universitätsmedizin, Berlin, Hessische Strasse 3–4, D-10115 Berlin, Germany. E-mail elena.kaschina@charite.de

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type 2 receptors (AT₂Rs), which counteract AT₁R actions, thus promoting antiproliferation, antiproliferation, and antifibrosis.⁶

AT₂Rs are upregulated under experimental MI⁷ and in human heart with interstitial fibrosis,⁸ although in patients with HF, AT₂R expression may be differently regulated.⁹ Cardiac AT₂R overexpression attenuates LV remodeling after MI in transgenic mice,^{10,11} whereas AT₂R blockade impairs cardiac function after MI in rats.¹² The recently developed nonpeptide AT₂R agonist compound 21 (C21) allows direct, selective, and specific AT₂R stimulation.^{13,14}

We have recently demonstrated a profound improvement of cardiac function 7 days after MI by direct AT₂R stimulation.¹⁵ Beneficial effects were based on anti-inflammatory, antiapoptotic, and antioxidative mechanisms. Importantly, C21 led to a significant attenuation of scar elongation after MI, pointing to a favorable effect of AT₂R activation on post-MI cardiac remodeling.¹⁵ Furthermore, C21 prevented vascular remodeling in L-NAME (*N*^G-nitro-L-arginine methyl ester) hypertension¹⁶ and stroke-prone spontaneously hypertensive rats,¹⁷ and it preserved renal integrity by preventing inflammatory cell infiltration and collagen accumulation.¹⁸ However, it has not been investigated yet whether chronic AT₂R stimulation has any effect on cardiac remodeling and resulting HF after MI.

The present study was designed to assess the long-term effects of treatment with the nonpeptide AT₂R agonist C21 on cardiac function and structural myocardial alterations 6 weeks after MI. We studied the potential underlying mechanisms of AT₂R activation on the heart by exploring extracellular matrix turnover processes of the postischemic myocardium, especially with regard to the tissue inhibitor of metalloprotease 1 (TIMP1) and matrix metalloprotease 9 (MMP9) axis.

Methods

Animals

This study was performed in strict accordance with National and European guidelines for animal experiments with approval by the ethics commission of the regulatory authorities of the City of Berlin, Germany, the Landesamt fuer Gesundheit und Soziales (registration number G0307/06).

Experimental Protocol

MI was induced by ligation of proximal left anterior descending coronary artery as previously described.¹⁵ Male Wistar rats were randomly assigned to the following groups: C21 treatment (0.03 mg/kg daily IP), vehicle treatment (0.9% NaCl), and sham-operated controls. Treatment was started 6 hours after MI. Animals with ejection fraction <35% were excluded from the experiment before assignment to groups. C21 was kindly provided by Vicore Pharma (Gothenburg, Sweden).

Analysis of Hemodynamic Parameters

Transthoracic Doppler echocardiography (M-mode and Doppler measurements) was performed 1 and 6 weeks after MI in rats anesthetized with isoflurane (2%) with the use of the high-resolution imaging system Vevo 770 (VisualSonics, Canada), and invasive hemodynamic assessment was performed at the end of the study using a fiber-optic pressure transducer catheter (Samba Sensors, Sweden) and Chart5 software for analysis as previously described.^{15,19}

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction, Western Blot Analysis, and ELISA

Quantitative real-time reverse transcription polymerase chain reaction for measurement of MMP9, MMP2, and transforming growth

factor β 1 (TGF- β 1) mRNA in heart tissues and TGF- β 1 in cardiac fibroblasts (CFs) in vitro, as well as immunoblotting for MMP2, MMP9, TGF- β 1, and TIMP1 protein expression in LV, was performed according to standard protocols. TGF- β 1 concentration in plasma was determined by ELISA. TGF- β 1 expression in LV was studied immunohistochemically. Details of primers and antibodies can be found in the online-only Data Supplement.

Histology and Hydroxyproline Content in the Heart

Paraffin-embedded cross-sections of the heart were stained for collagen (sirius red/picric acid). Myocyte diameter was quantified by means of computer-aided histomorphometry by using of a $\times 40$ objective ($\times 60$ on monitor). The diameters were determined on a minimum of 30 myocytes from each animal ($n=4$) in noninfarcted regions. Interstitial fibrosis was quantified by means of computer-aided histomorphometry (magnification $\times 60$). Results were calculated as percentage of sirius red–positive areas in randomly chosen pictures from cardiac sections. The amount of collagen in the LV was analyzed by quantification of the tissue hydroxyproline content using the modified method based on alkaline hydrolysis as previously described.^{16,19}

Cell Culture Experiments

Primary rat CFs were isolated as described by Agocha et al.²⁰ CFs were incubated with interleukin 1 α (IL-1 α ; 20 ng/mL, Sigma) in the presence or absence of C21 (0.25, 0.5, 1 μ mol/L) and PD123319 (10 μ mol/L). MMP2 and MMP9 activity was analyzed by gelatin zymography as described previously.^{21,22} mRNA expression of MMP9, MMP2, and TGF- β 1 was evaluated by quantitative real-time reverse transcription polymerase chain reaction.

Fluorescence Microscopy

MMP9 and TIMP1 release was studied by fluorescence microscopy (Biorevo BZ-9000, Keyence Japan) in single CFs after stimulation with IL-1 α (20 ng/mL) in the presence or absence of C21 (1 μ mol/L) for 1, 4, 24, and 48 hours. Antibodies are specified in the online-only Data Supplement.

Statistical Analysis

Results are expressed as mean \pm SEM. Multiple comparisons were analyzed with 1-way ANOVA followed by the Bonferroni post hoc test. Two-group comparisons were analyzed by the 2-tailed Student unpaired *t* test for independent samples. Differences were considered statistically significant at a value of $P<0.05$.

Results

C21 Improves Systolic and Diastolic Cardiac Functions 6 Weeks After MI

Postinfarct mortality was 31%. Animal losses all occurred within the first 6 hours after MI before animals were assigned to the respective treatment groups.

Echocardiographic outcomes obtained 6 weeks after MI are presented in the Table. MI induced a significant LV dilation reflected by increased systolic LV inner diameter ($P<0.05$). Systolic function was impaired as evidenced by a decrease in LV ejection fraction ($P<0.001$), fractional shortening ($P<0.01$), and stroke volume ($P<0.05$). MI also deteriorated LV diastolic function, leading to an increase in the ratio of early (E) to late (A) ventricular filling velocities (E/A ratio; $P<0.05$) and shortening of E-wave deceleration time ($P<0.01$; Table).

Chronic treatment with C21 significantly improved systolic function, raising ejection fraction by 17.8% ($P<0.001$) and fractional shortening by 9.9% ($P<0.05$) versus vehicle. C21

Table. Hemodynamic Variables in Wistar Rats Measured 6 Weeks After MI by Transthoracic Doppler Echocardiography

Hemodynamic Parameter	C 21		
	Sham	Vehicle	0.03 mg/kg per day
EF, %	74.6±2.51	53.4±7.03*	71.2±4.74†
FS, %	50.5±5.19	30.9±3.08‡	40.8±2.32§
LVIDd, mm	7.9±0.14	8.7±0.63	7.8±0.54§
LVIDs, mm	4.7±0.47	5.2±0.76	4.4±0.48§
E/A	1.8±0.17	2.3±0.13	1.9±0.09§
EDT, ms	17.8±2.29	12.4±0.78‡	15.6±1.61
Stroke volume, μ L	44.3±5.37	35.6±1.42	39.4±2.95
Heart rate, bpm	339.3±11.84	357.8±6.46	335.3±6.29

E/A indicates ratio between early (E) and late (atrial [A]) ventricular filling velocity; EDT, deceleration time of early filling wave; EF, ejection fraction; FS, fractional shortening; LVIDd, diastolic left ventricular inner diameter; LVIDs, systolic left ventricular inner diameter; and MI, myocardial infarction.

* $P<0.001$ vs sham; † $P<0.001$ vs vehicle; ‡ $P<0.01$; § $P<0.05$; || $P<0.05$.

also reduced systolic LV inner diameter from 5.2 ± 0.7 mm in vehicle-treated to 4.4 ± 0.4 mm ($P<0.05$) in C21-treated rats and diastolic LV inner diameter from 8.7 ± 0.6 to 7.8 ± 0.5 mm ($P<0.05$), respectively. C21 further improved cardiac wall motion (Figure S1A in the online-only Data Supplement). Improvement of diastolic function by C21 was evidenced by a 17.4% decrease in E/A ratio ($P<0.05$; versus vehicle; Table; Figure S1B).

Hemodynamic parameters measured via intracardiac Samba catheter are presented in Figure 1. Rats with MI developed systolic dysfunction, as evidenced by decreased contractility index (125.4 ± 4.2 versus sham 142.3 ± 2.0 L/s; $P<0.05$; Figure 1A) and maximal peak rate of LV pressure increase (dP/dt_{\max} : 9364 ± 369 versus sham 11959 ± 420 mm Hg/s; $P<0.05$; Figure 1B).

Rats with MI also exhibited a severe diastolic dysfunction, as defined by elevated LV end-diastolic pressure (22.1 ± 1.4 versus sham 14.4 ± 1.0 mm Hg; $P<0.05$; Figure 2D), increased minimal peak rate of LV pressure increase (dP/dt_{\min} : -10262 ± 464 versus sham -12524 ± 529 mm Hg/s; $P<0.05$; Figure 1E), and reduced diastolic duration time ($P<0.01$; Figure 1F).

C21 significantly improved systolic cardiac function, enhancing contractility index (141.7 ± 4.2 versus vehicle 125.4 ± 4.2 L/s; $P<0.05$) and dP/dt_{\max} (11540 ± 594 mm Hg/s versus 9364 ± 369 mm Hg/s; $P<0.05$; Figure 1A and 1B) and reducing LV maximal pressure ($P<0.05$; Figure 1C).

C21 demonstrated favorable effects on the diastolic function by reducing LV end-diastolic pressure (16.9 ± 1.2 versus vehicle 22.1 ± 1.4 mm Hg; $P<0.05$) and dP/dt_{\min} (-11805 ± 398 versus vehicle -10262 ± 464 mm Hg/s; $P<0.05$; Figure 1D and 1E) and by increasing diastolic duration time ($P<0.05$; Figure 1F).

To assess indirect parameters of arterial stiffness, pulse wave analysis was performed on pressure curves obtained from the ascending aorta. The augmentation index in vehicle-treated animals was increased (25.4 ± 1.2 versus sham 15.57 ± 1.3 ; $P<0.05$), and C21 attenuated this increase (17.8 ± 1.2 versus vehicle 25.4 ± 1.2 ; $P<0.05$).

Myocyte Diameter, Collagen Content, and Expression of TGF- β 1 in the Heart 6 Weeks After MI

Myocyte diameter was significantly increased 6 weeks post-MI (12.57 ± 1.85 versus sham 10.56 ± 1.50 μ m; $P<0.01$), and C21 treatment further increased the diameter of myocytes (15.38 ± 1.30 versus vehicle 12.57 ± 1.85 μ m; $P<0.001$; Figure S2).

Less collagen accumulation was found in the heart after C21 treatment as demonstrated by staining with the collagen-specific dye sirius red (Figure S3). Interstitial fibrosis was markedly increased in the noninfarcted region of vehicle-treated post-MI animals ($5.5\pm 0.3\%$ versus sham $2.8\pm 0.3\%$; $P<0.01$); treatment with C21 completely prevented this effect ($3.0\pm 0.2\%$ versus vehicle $5.5\pm 0.3\%$; $P<0.001$; Figure S4A).

Collagen content was studied by measuring tissue hydroxyproline concentration. Hydroxyproline increased by 24% ($P<0.01$) post-MI compared with sham group, whereas C21 attenuated this increase ($P<0.05$; Figure S4B).

Because fibrosis is promoted by TGF- β 1, its expression was investigated in the peri-infarcted zone. In the vehicle group, TGF- β 1 was strongly enhanced in fibrotic tissues and cardiomyocytes (Figure 2A). C21 prevented the overexpression of TGF- β 1.

Accordingly, TGF- β 1 expression in the LV, which was significantly upregulated both on the mRNA (1.3-fold; $P<0.05$) and particularly on protein levels (40-fold; $P<0.01$) post-MI, was completely abolished by C21 ($P<0.01$; Figure 2B and 2C).

Furthermore, serum concentration of active TGF- β 1 tended to be elevated 6 weeks post-MI, whereas the latent form of TGF- β 1 was decreased ($P<0.05$; Figure 2D and 2E). C21 treatment normalized the levels of both forms ($P<0.05$).

Regulation of TIMP1, MMP2, and MMP9 by C21 in the LV 6 Weeks After MI

As demonstrated in Figure 3A and 3B, MMP9 was upregulated post-MI both at the mRNA (2.9-fold; $P<0.05$) and protein levels (3.6-fold; $P<0.001$). C21 treatment significantly attenuated this increase.

MMP2 protein but not mRNA was also upregulated (Figure S5). Zymographic analysis of the LV post-MI extracts demonstrated an increased release of active MMP9 (Figure 3C and 3D). Again, C21 significantly prevented MMP9 secretion, whereas there was no statistically significant effect on MMP2 activity (Figure 3E). Protein expression of TIMP1, the native inhibitor of MMP9, was lower after MI (Figure 3F). C21 strongly reversed this decrease (17.7-fold; $P<0.01$) and, in turn, attenuated the increased MMP9/TIMP1 ratio (Figure 3G).

Regulation of TIMP1, MMP2, MMP9, and TGF- β 1 Expression in Rat Primary CFs

Zymographic analysis of MMP9 and MMP2 activity in CFs is presented in Figure 4. C21 prevented the IL-1 α -stimulated secretion of MMP9 in a concentration-dependent manner, whereas MMP2 release was not influenced. AT₂R blockade with PD123319 thoroughly abolished the effect of C21 on the

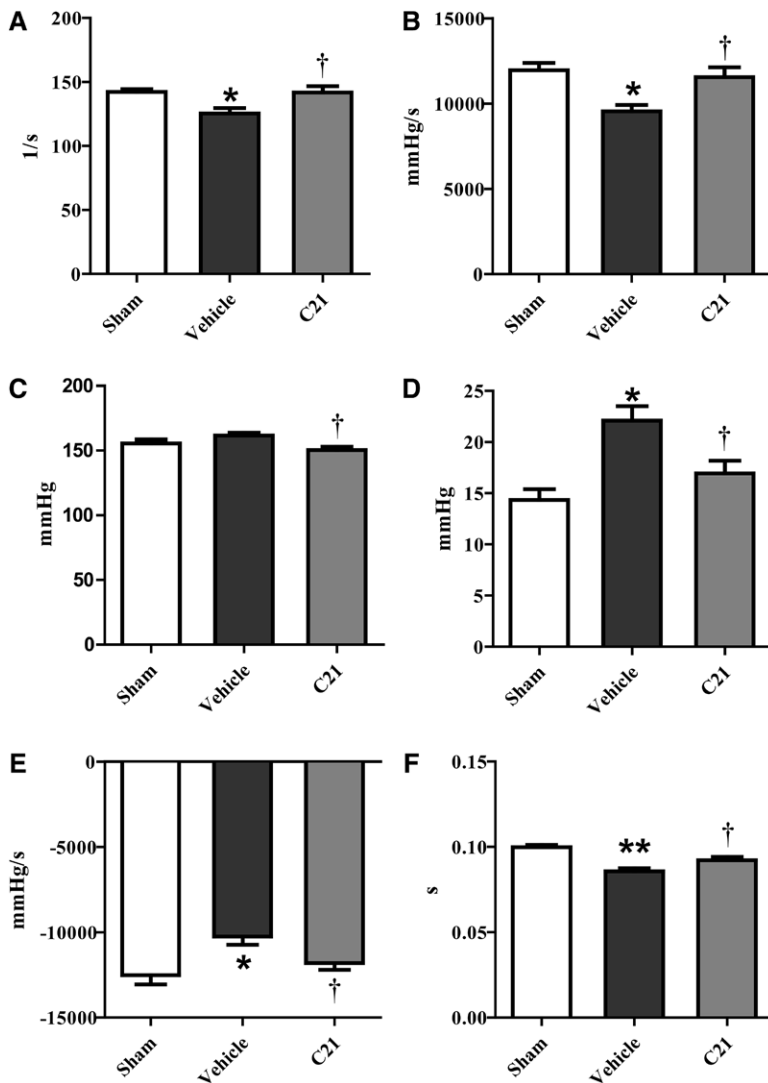


Figure 1. Hemodynamic parameters measured in left ventricle (LV) by intracardiac Samba catheter 6 weeks after myocardial infarction. **A**, Contractility index. **B**, Maximal peak rate of LV pressure increase (microvascular permeability index, dP/dt_{max}). **C**, LV maximal pressure. **D**, LV end-diastolic pressure. **E**, Minimal peak rate of LV pressure increase (dP/dt_{min}). **F**, Diastolic duration; ** $P < 0.01$, * $P < 0.05$ vs sham; † $P < 0.05$ vs vehicle; (n=8 in each group, mean±SEM); 1-way ANOVA.

IL-1 α -induced MMP9 secretion (Figure S6). C21 also significantly attenuated TGF- β 1 mRNA upregulation (Figure S7).

The results of TIMP1 and MMP9 immunostaining in CFs are presented in Figure 5. After stimulation with IL-1 α , MMP9 (red stain) was expressed in cytoplasm already at 4 hours and reached its maximum at 48 hours. TIMP1 (green stain) appeared 24 hours after stimulation, was colocalized (yellow stain) with MMP9, and disappeared at 48 hours. In C21-treated cells, TIMP1 was strongly expressed already 4 hours after IL-1 α stimulation, reached maximum at 24 hours, and was distinguishable until 48 hours in a colocalization with MMP9. In C21-treated cells, even at 48 hours after stimulation not linked MMP9 (red stain) was undistinguishable.

Discussion

This study describes protective effects of long-term selective AT_2 R stimulation with the AT_2 R agonist C21 in a chronic model of MI. C21 improved both systolic and diastolic heart functions, largely prevented post-MI LV dysfunction, and reduced arterial stiffness.

The present study is in line with our previous data obtained 7 days after MI, which showed an improvement of cardiac

function in the acute phase after MI. In this earlier study, the favorable effect of AT_2 R stimulation with C21 on cardiac function was prevented by the AT_2 R antagonist, PD123319, confirming AT_2 R specificity of C21 actions.¹⁵ Hemodynamic data are also in agreement with recent investigations performed in an AT_2 R overexpression model, where the AT_2 R protected the heart from ischemic injury 4 weeks post-MI.²³ However, in a study looking at cardiac function and remodeling in a model of MI induced by coronary occlusion-reperfusion in mice, no attenuation of post-MI LV remodeling by a 4-week treatment with C21 was observed.²⁴ The lack of treatment effect of C21 in this study was probably because of the application of the same dose of C21 that is usually used for bolus injections (0.3 mg/kg bw) by minipump for 24 hours, which considering a plasma half-life of C21 of maximum 4 hours results in too low plasma levels and thus in underdosing.

In the late phase post-MI, adverse postischemic cardiac remodeling is characterized by fibrotic processes and collagen deposition in the remote myocardium, which have the potential of increasing LV mass at the cost of LV function.²⁵ Therefore, the role of AT_2 R stimulation in post-MI cardiac

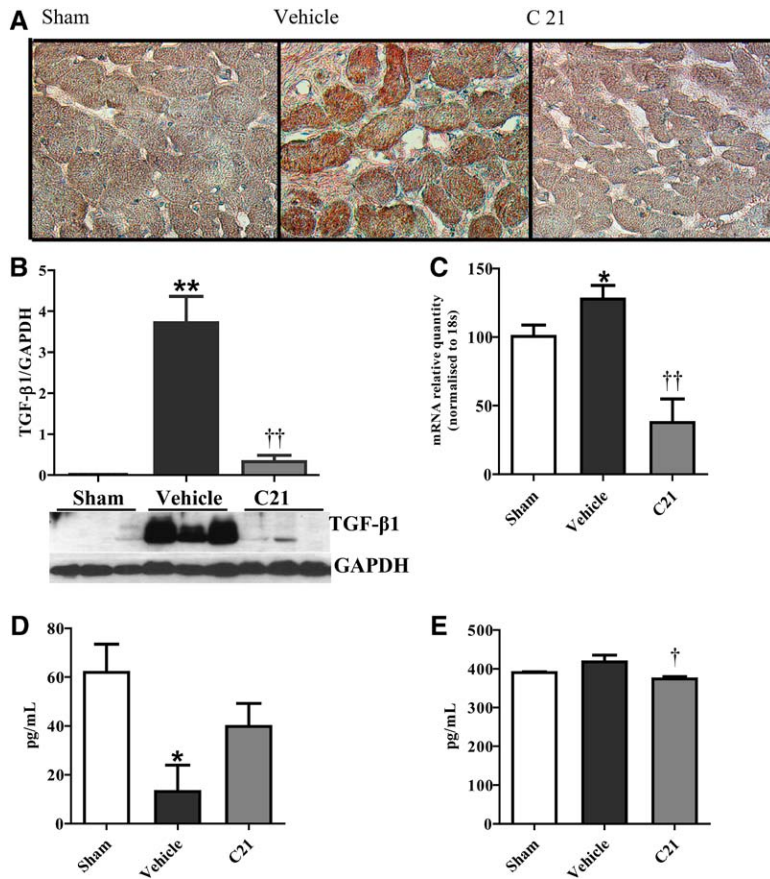


Figure 2. Transforming growth factor (TGF) β 1 expression in left ventricle (LV) 6 weeks after myocardial infarction. **A**, Immunostaining of TGF- β 1, red stain, $\times 40$. **B**, Western blot analysis of TGF- β 1. Densitometric data of proteins are mean \pm SEM (n=6). **C**, Relative expression of TGF- β 1 mRNA in LV (n=6). **D**, Concentration of latent TGF- β 1 in serum (n=6). **E**, Concentration of active TGF- β 1 in serum (n=6); ** P <0.01, * P <0.05 vs sham; †† P <0.01, † P <0.05 vs vehicle; 1-way ANOVA.

fibrosis and the underlying mechanisms were the focus of this study.

We found that C21 decreased interstitial fibrosis and collagen accumulation in the remote myocardium after ischemia. In parallel, C21 reduced the increased augmentation index, which characterizes arterial stiffness and correlates with vascular fibrosis, as well as with the severity of LV hypertrophy and coronary heart disease.²⁶ These data are in agreement with previous reports demonstrating an antifibrotic effect of the AT₂R agonist in the aorta of L-NAME-treated hypertensive rats¹⁶ and in the vessels and heart of SHR.¹⁷

Interestingly, C21 administration reinforced post-MI compensatory hypertrophy of cardiac myocytes and by this mechanism possibly contributed to functional improvement of the heart.

A similar trend was seen by Voros et al,²⁷ but in their study did not reach statistical significance probably because of limited power of the study as the authors state in the discussion.

We further investigated the effect of AT₂R stimulation on TGF- β 1 expression, a profibrotic cytokine that is extensively expressed after ischemia and is upregulated by angiotensin II via AT₁R activation in cardiac myocytes and fibroblasts.^{28,29} This study is the first to demonstrate that AT₂R stimulation prevents the increase in TGF- β 1 expression after MI, both at protein and transcriptional levels. The elevated serum concentration of active TGF- β 1 was also decreased by C21. In vitro experiments in primary CFs further demonstrated that C21 attenuated the IL-1 α -induced increase in TGF- β 1 mRNA expression, suggesting its regulation through a direct effect of AT₂R stimulation on the regulation of TGF- β 1 expression.

The degradation of matrix components by MMPs and the resulting side-by-side slippage of cardiomyocytes are other mechanisms that may contribute to LV wall thinning in the remote region after MI.²⁵ It is, therefore, suggested that the interplay of MMPs and their regulators is important in the development of myocardial fibrosis and post-MI LV dysfunction and that HF can be modified by modulating MMP activity.

Gelatinases MMP2 and MMP9 have been shown to be main regulators of ECM (extracellular matrix) in cardiac tissue because they possess substrate affinity for denatured fibrillar collagen and exhibit proteolytic activity against elastin and proteoglycans.³⁰

MI induced the proteolytic activity of MMP9 in the LV, which was attenuated by C21 treatment. Similarly, C21 dose dependently decreased gelatinolytic activity of MMP9 in CFs. Furthermore, C21 regulated the expression of TIMP1, the natural inhibitor of MMP9. Deficiency of TIMP1 is known to promote LV dilatation and increase LV end-diastolic volume³¹ and has also been demonstrated to exacerbate LV remodeling after MI in mice after MI.³²

In our study, the higher concentration of MMP9 and the increased MMP9/TIMP1 protein expression ratio in cardiac tissue post-MI evidenced the proteolytic dysbalance, which was ameliorated by treatment with C21. The molecular mechanisms, by which the AT₂R inhibits proteolysis, are complex and require further investigation, but several possible interactions exist.

First, the AT₂R seems to be involved in the interplay between MMPs and TGF- β 1. Increasing evidence points toward the

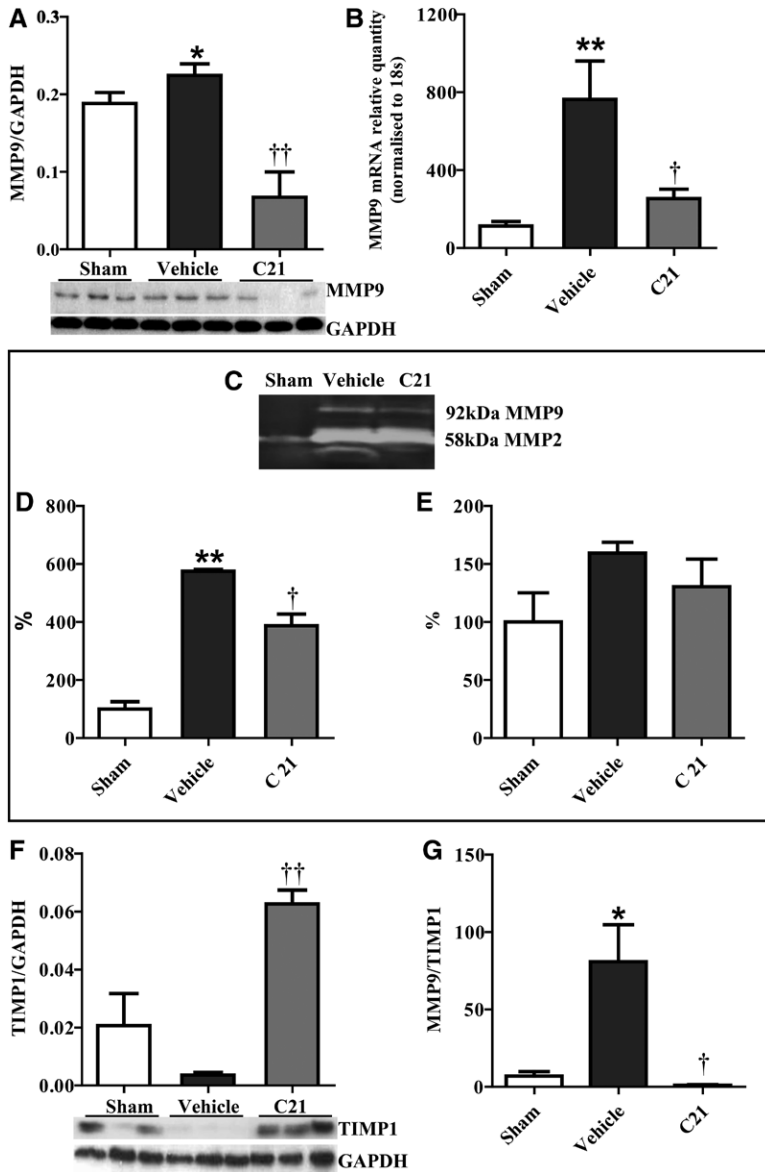


Figure 3. Matrix metalloproteinase 9 (MMP9; **A**) and tissue inhibitor of matrix metalloproteinase 1 (TIMP1; **F**) protein expression and MMP9/TIMP1 ratio (**G**) in left ventricle 6 weeks after myocardial infarction. Representative images and densitometric data of proteins are mean±SEM (n=6). **B**, MMP9 mRNA. **C**, Representative gelatin zymogram loaded with heart tissue extracts. Semiquantitative analysis of MMP9 (**D**) and MMP2 (**E**) activity in heart tissues measured by gelatin zymography (n=3). ***P*<0.01, **P*<0.05 vs sham; ††*P*<0.01, †*P*<0.05 vs vehicle; 1-way ANOVA.

possibility that TGF-β1 increases MMP activity within the myocardium.²⁹ However, MMP9 and MMP2 are both able to cleave latent TGF-β1, releasing its active form,³³ which in turn

activates transcription of TIMPs.³⁴ This suggests that a feed-forward loop could develop in cardiac remodeling, in which the roles of MMPs and TGF-β1 are intertwined.

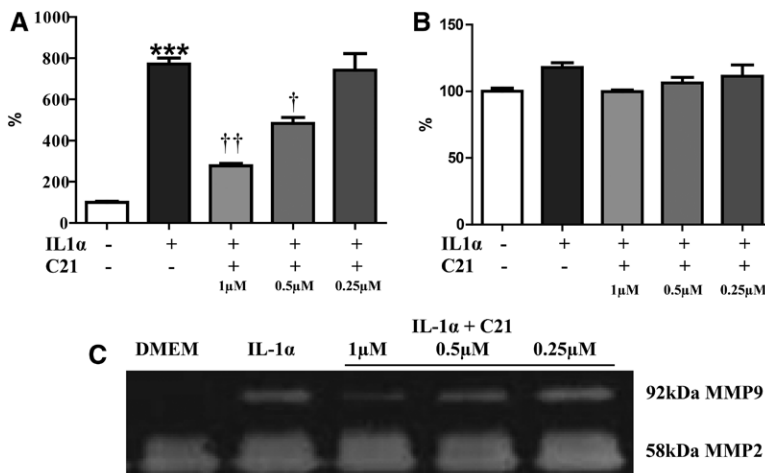


Figure 4. Effect of compound 21 (C21) on cytokine-induced matrix metalloproteinase 9 (MMP9) and MMP2 secretion in cardiac fibroblasts (CFs). Activity of MMP9 (**A**) and MMP2 (**B**) in CFs determined via gelatin zymography. Bars represent densitometric data of 3 independent experiments (mean±SEM). **C**, Representative gelatin zymogram loaded with conditioned media from CFs. ****P*<0.001 vs sham; ††*P*<0.01, †*P*<0.05 vs vehicle; 1-way ANOVA.

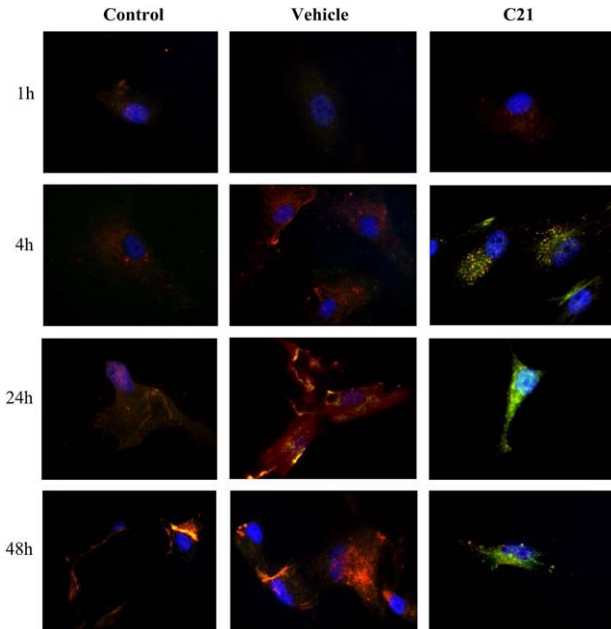


Figure 5. Representative images of single cardiac fibroblasts show time-dependent changes in protein expression of matrix metalloproteinase 9 (MMP9) and tissue inhibitor of matrix metalloproteinases 1 (TIMP1) after interleukin 1 α stimulation. Treatment with compound 21 (C21) induced the earliest expression of TIMP1 (4 hours) and a diminished MMP9 expression at all time points (1, 4, 24, 48 hours). Cells were stained with TIMP1 antibodies (fluorescein isothiocyanate, green), MMP9 antibodies (Cy3, red), and Hoechst (blue) for nuclei counterstaining. Colocalization of TIMP1 with MMP9 is stained with yellow. The images were acquired by fluorescence microscope (Biorevo BZ-9000; Keyence, $\times 60$).

TIMP1 activation may contribute to antiproteolytic effects of C21 in the heart. Our immunostaining experiments performed in single CFs and determining the time course of AT₂R-induced expression of TIMP1 and MMP9 suggest that C21 in a first step activates TIMP1 followed by inhibition of MMP9. TIMP1 is also known to possess antiapoptotic activity,³⁵ and this fact is in accordance with antiapoptotic properties of C21 demonstrated previously.^{15,36} Thus, TIMP1 may be a major mediator of the cardioprotective effects of C21. Given that MMPs generally play a pivotal role in extracellular matrix degradation, the antiproteolytic effect of C21 might be of great relevance for tissue remodeling in various pathologies.

It is a limitation of this study that we did not have an animal group treated with C21 plus an AT₂R antagonist (PD123319) to prove AT₂R specificity of the observed protective effects of C21. However, AT₂R specificity of a beneficial effect of C21 on post-MI cardiac performance has been shown by us in a previous study with shorter duration.¹⁵ Furthermore, we demonstrate in this study in *in vitro* experiments that the inhibitory effect of C21 on MMP activity, which is an entirely new finding, is AT₂R specific, that is, reversible by PD123319.

Perspectives

This study demonstrates that long-term stimulation of the AT₂R with C21 improved postischemic cardiac function by preventing adverse myocardial remodeling and reducing proteolysis. Furthermore, C21 activated TIMP1, inhibited

MMP9, and decreased TGF- β 1 expression as well as collagen accumulation in the heart.

This study is of clinical relevance because it supports the hypothesis that AT₂R stimulation could represent a novel therapeutic concept in postischemic LV remodeling and HF treatment.

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Disclosures

B. Dahlöf and T. Unger received speaker fees from Vicore Pharma. T. Unger has a modest interest in Vicore Pharma. U.M. Steckelings received modest research support from Vicore Pharma. B. Dahlöf has a significant interest in Mintage Scientific, the owner of Vicore Pharma. The other authors report no conflicts.

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Novelty and Significance

What Is New?

- Angiotensin type 2 receptor (AT₂R) stimulation by the selective nonpeptide agonist compound 21 improved systolic and diastolic functions in the late stage post–myocardial infarction in rats.
- AT₂R stimulation prevented fibrosis of peri-infarct myocardium and improved arterial stiffness parameters.
- The main cellular mechanisms underlying cardiac protection by AT₂R stimulation include antiproteolysis via the activation of tissue inhibitor of matrix metalloproteinase 1 and inhibition of matrix metalloproteinase 9, as well as antifibrosis via decreased transforming growth factor β1 expression in the heart.

What Is Relevant?

- AT₂R stimulation by compound 21 improves cardiac function in the late stage post–myocardial infarction and ameliorates left ventricular remodeling.

Summary

AT₂R stimulation could represent a novel therapeutic concept in postischemic left ventricular remodeling and heart failure treatment.

Angiotensin Type 2 Receptor Stimulation Ameliorates Left Ventricular Fibrosis and Dysfunction via Regulation of Tissue Inhibitor of Matrix Metalloproteinase 1/Matrix Metalloproteinase 9 Axis and Transforming Growth Factor β 1 in the Rat Heart

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**ANGIOTENSIN TYPE 2 RECEPTOR STIMULATION AMELIORATES
LEFT VENTRICULAR FIBROSIS AND DYSFUNCTION VIA
REGULATION OF TIMP1/MMP9 AXIS AND TGF- β 1 IN THE RAT
HEART**

Dilyara Lauer¹, Svetlana Slavic^{1,2}, Manuela Sommerfeld¹, Christa Thöne-Reineke^{1,3}, Yuliya Sharkovska⁴, Anders Hallberg⁵, Bjorn Dahlöf⁶, Ulrich Kintscher¹, Thomas Unger^{1,7}, Ulrike Muscha Steckelings^{1,8*}, Elena Kaschina^{1*}

¹Center for Cardiovascular Research (CCR) and Institute of Pharmacology, Charité – Universitätsmedizin, Berlin, Germany; ²Institute of Physiology, Pathophysiology and Biophysics, Department of Biomedical Sciences University of Veterinary Medicine, Vienna, Austria; ³Forschungseinrichtung für Experimentelle Medizin, Charité – Universitätsmedizin Berlin, Germany; ⁴Institute of vegetative Anatomy, Charité – Universitätsmedizin, Berlin, Germany; ⁵Department of Medicinal Chemistry, Uppsala University, Sweden; ⁶Sahlgrenska University Hospital, Gothenburg, Sweden; ⁷CARIM - School for Cardiovascular Diseases Maastricht University, Maastricht, The Netherlands; ⁸IMM – Department of Cardiovascular and Renal Research, University of Southern Denmark, Odense, Denmark

* Both authors contributed equally

Short title: AT2-receptor in post-MI cardiac remodeling

Corresponding author: Elena Kaschina, Center for Cardiovascular Research (CCR) and Institute of Pharmacology, Charité – Universitätsmedizin, Berlin, Hessische Strasse 3-4, D-10115 Berlin, Germany; phone: +49-30-450-525-024; fax: +49-30-450-525-901;

e-mail: elena.kaschina@charite.de.

Expanded Materials and Methods

Animals

Male normotensive Wistar rats (weight 200 to 220g; Harlan Winkelmann, Borchon, Germany) were kept in a specific pathogen-free barrier under standardized conditions with respect to temperature ($23\pm 1^\circ\text{C}$) and humidity and were housed on a 12-hour light/dark cycle in groups of 5 animals with food and water ad libitum.

Myocardial infarction

Under anaesthesia with ketamine/xylazine (80/10 mg/kg i.p.) rats were intubated and connected with a small-animal ventilator with room air at a rate of 75 cycles/min and 3.5mL tidal volume. Left lateral thoracotomy was performed, and a suture was tightened around the proximal left anterior descending coronary artery. Sham-operated rats underwent the same surgical procedure with the exception of the coronary ligation. Analgesic Flunixin-Meglumine (1,0 mg/kg i.p.) was applied 45 min before anesthesia and daily 3 days after operation.

The number of animals per group was 12 in C21 and vehicle treated and 8 in sham operated groups at the time of euthanasia. C21 was dissolved in 0,9%NaCl and administered 0,03mg/kg daily i.p.

Western Blot Analysis

Protein lysates were prepared according to standard protocols. Protein expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Immunoreactive bands were detected by enhanced chemiluminescence (GE Health Care, Germany) and quantified with ImageJ software.

Antibodies for Western Blot analysis

Antibodies	Source	Dilution	Manufacturer	
Primary antibodies	MMP-2	Polyclonal rabbit	1:1000	Abcam
	MMP-9	Polyclonal rabbit	1:1000	Abcam
	TIMP-1	Polyclonal rabbit	1:500	Santa Cruz
	TGF- β	Polyclonal rabbit	1:500	Santa Cruz
	GAPDH	Monoclonal mouse	1:500	MerckMillipor
Secondary antibodies	Anti-mouse	Polyclonal goat	1:2000	Dako
	Anti-rabbit	Polyclonal goat	1:2000	Dako

Antibodies for fluorescence microscopy

Antibodies	Source	Dilution	Manufacturer	
	MMP-2	Polyclonal mouse	1:200	Santa Cruz
	MMP-9	Polyclonal mouse	1:200	Santa Cruz
	TIMP-1	Polyclonal rabbit	1:200	Santa Cruz
	TGF- β	Polyclonal rabbit	1:200	Santa Cruz
Secondary antibodies	anti-mouse Cys3	Polyclonal mouse	1:250	Dianova
	anti-mouse FITC	Polyclonal rabbit	1:250	Dianova
	anti-rabbit	Polyclonal goat	1:200	DAKO

Immunohistochemistry

Immunohistochemistry of TGF- β 1 was performed in paraffin-embedded tissue sections (5 μ m) using the avidin-biotin complex method according to the manufacturer's instructions (Vectastain ABC; Vector Laboratories, Burlingame, California, USA). Peroxidase activity was visualized by 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Primary antibodies against TGF- β 1 (1:50, Santa Cruz Biotechnology Inc., Heidelberg, Germany) were used.

mRNA analysis

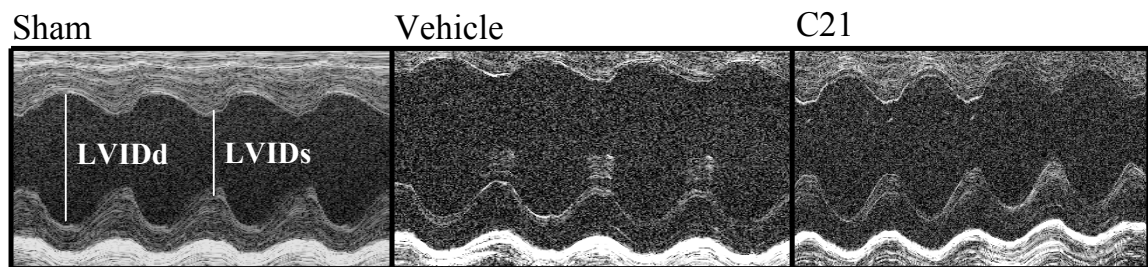
Total RNA from LV tissue and CFs was isolated by TRIZOL reagent (Invitrogen) and subsequently treated with DNase-I (Promega). qRT-PCR was performed with MX3005p QPCR System (Stratagene) using SYBR green reaction mix and primers indicated in supplement. All samples were measured in triplicate, and expression values were normalized to 18s rRNA. Data analysis was done using the MxProTM ET QPCR software (Stratagene) and $\Delta\Delta$ Ct-method.

Primers

Gene (Rat)	Primer sequences for SYBR Green® real-time PCR
18s	Forward: 5'- GGGAGGTAGTGACGAAAAATAACAAT-3' Reverse: 5'- TTGCCCTCCAATGGATCCT-3'
MMP 2	Forward: 5'- GACGCTGGGAGCATGGAG-3' Reverse: 5'- TTACGCGGACCACTTGTCC-3'
MMP 9	Forward: 5'- CTTCTCTGGGCGCAAAATG-3' Reverse: 5'- CCGGTGACCAGGGTTACCT-3'
TIMP1	Forward: 5'- TCTGGCATCCTCTTGTGCT-3' Reverse: 5'- CACAGCCAGCACTATAGGTCTTT-3'
TGF- β 1	Forward: 5'- GGTGGACCGCAACAACGCAATCTA-3' Reverse: 5'- GGGTGGCCATGAGGAGCAGGAA-3'

Figures

A



B

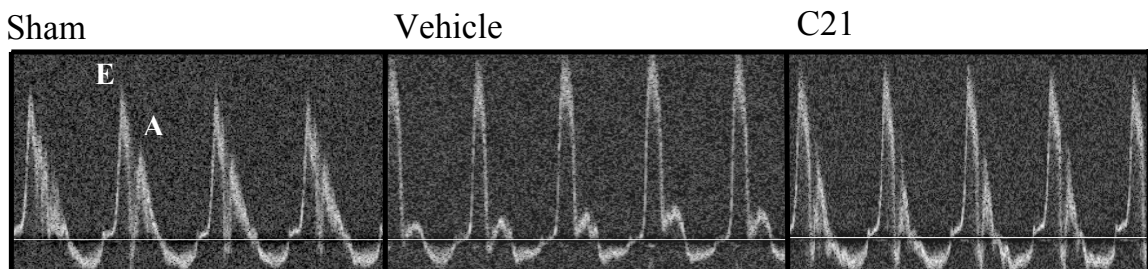


Figure S1. Effect of the C21 treatment on cardiac function 6 weeks post-MI. (A): M-mode echocardiograms of LV. Note LV dilatation, thinning and akinesis of the anterior wall in vehicle-treated rats. Diastolic (LVIDd) and systolic (LVIDs) inner dimensions are increased compared with sham-operated animals. C21 prevented LV dilation and improved anterior wall motion.

(B): Pulsed-wave Doppler spectra of mitral inflow. Note an augmentation of E-wave (early diastolic filling velocity), reduction of A-wave (atrial contraction filling velocity) in vehicle-treated rats vs. healthy rats. C21 reversed MI-induced alterations in E and A-waves almost back to sham level.

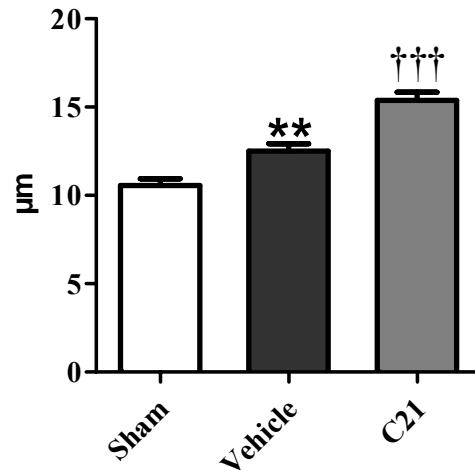


Figure S2: Myocyte diameter was quantified in cardiac sections by means of computer-aided histomorphometry, magnification x60. All of the values are given as mean±SEM; **p<0.01 versus sham group; †††p<0.001 versus vehicle; one-way ANOVA.

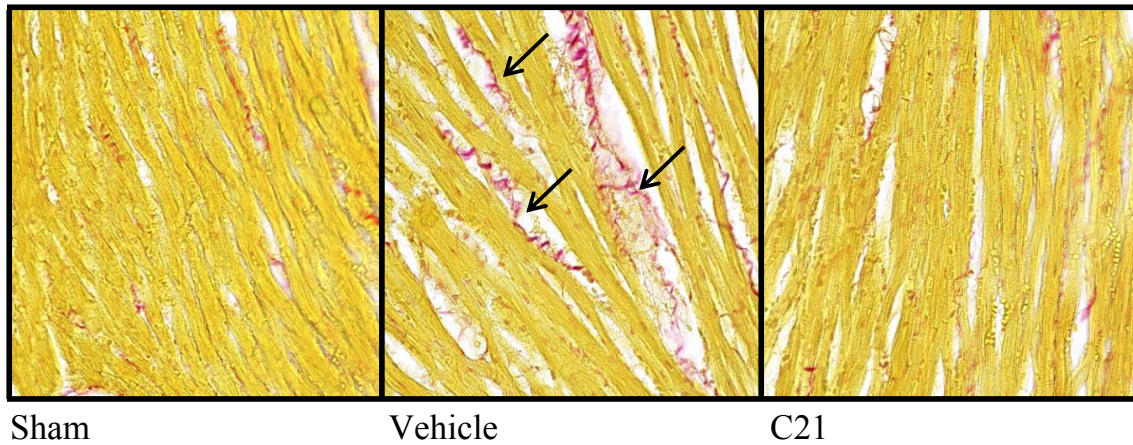


Figure S3: Representative cross-sections from LV 6 weeks after MI. Sirius red stain. Collagen fibers are stained red and cardiac myocytes are yellow, 40x. Note diffuse interstitial fibrosis in vehicle-treated animals which is partly prevented by C21 treatment.

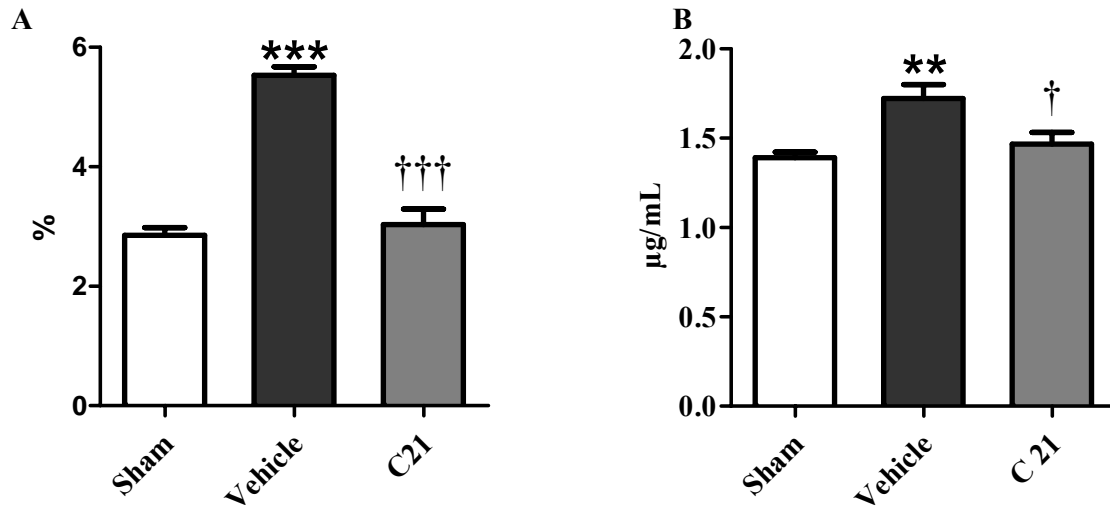


Figure S4: (A) Interstitial fibrosis was quantified in Sirius Red-stained cardiac sections by means of computer-aided histomorphometry, magnification x200. Results were calculated as percentage of Sirius-Red-positive area in randomly chosen pictures from cardiac sections. (B) Hydroxyproline concentration in septum 6 weeks after MI (n=3); All of the values are given as mean±SEM; ***p<0.001, **p<0.01 versus sham group; †††p< 0.001, †p<0.05 versus vehicle; one-way ANOVA.

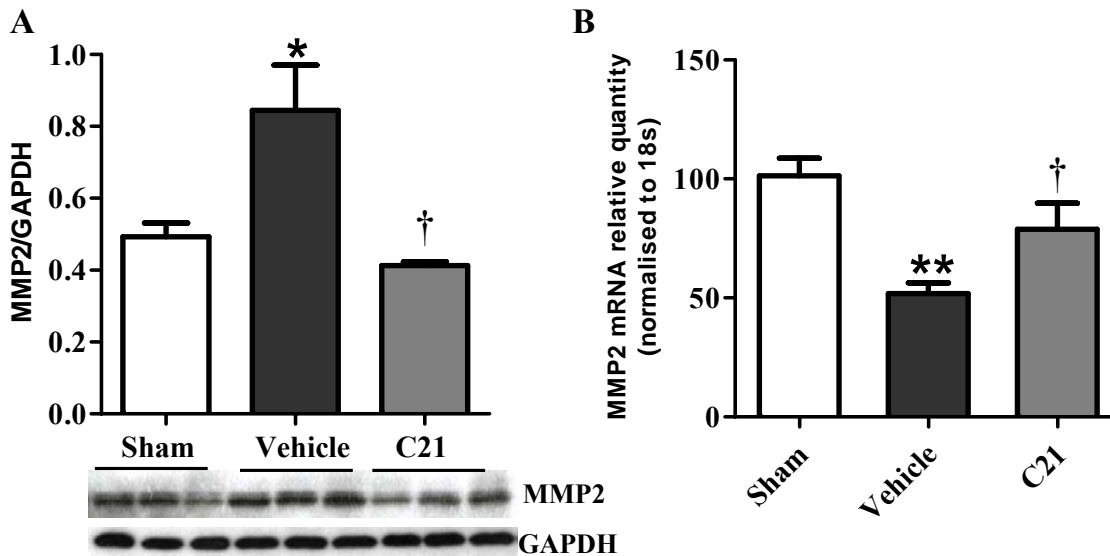


Figure S5: (A) MMP2 expression measured in LV 6 weeks post-MI. Western blot analysis (n=6). Representative image and densitometric data of proteins are mean ± SEM; (B): MMP2 mRNA. **p<0.01, *p<0.05 versus sham; †p<0.05 versus vehicle; one-way ANOVA.

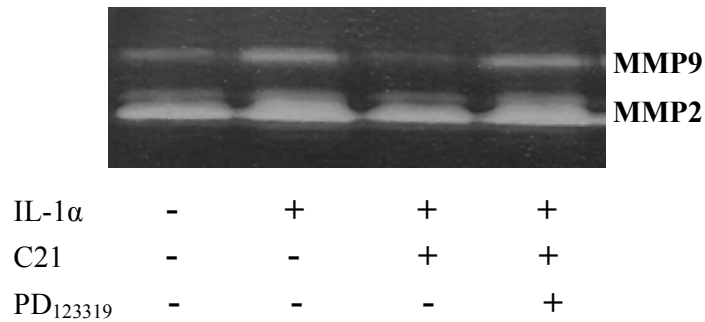


Figure S6. Effect of C21 and combination of C21 (1 μ M) plus PD 123319 (10 μ M) on cytokine-induced MMP9 and MMP2 secretion in CFs. Activity of MMP9 and MMP2 in CFs determined via gelatine zymography. PD 123319 abolished the effect of C21 on the IL-1 alpha induced MMP9 secretion.

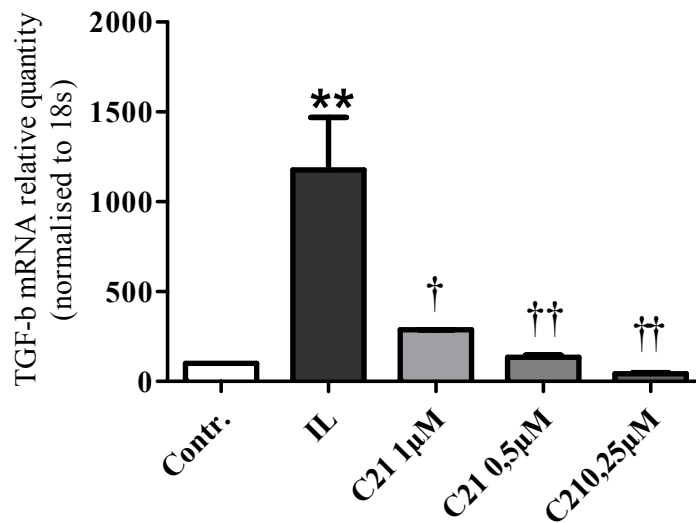


Figure S7. TGF- β 1 mRNA expression measured in primary rat cardiac fibroblasts. Bars represent densitometric data of three independent experiments (mean \pm S.E.M). C21 reduces IL 1 α -stimulated release of TGF- β 1 by cardiac fibroblasts. **p<0.01 versus sham; ††p<0.01, †p<0.05 versus vehicle; one-way ANOVA.