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Mechanisms underlying the cardiac antifibrotic effects of losartan metabolites

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Excessive myocardial collagen deposition and cross-linking (CCL), a process regulated by lysyl oxidase (LOX), determines left ventricular (LV) stiffness and dysfunction. The angiotensin II antagonist losartan, metabolized to the EXP3179 and EXP3174 metabolites, reduces myocardial fibrosis and LV stiffness in hypertensive patients. Our aim was to investigate the differential influence of losartan metabolites on myocardial LOX and CCL in an experimental model of hypertension with myocardial fibrosis, and whether EXP3179 and EXP3174 modify LOX expression and activity in fibroblasts. In rats treated with N^G-nitro-L-arginine methyl ester (L-NAME), administration of EXP3179 fully prevented LOX, CCL and connective tissue growth factor (CTGF) increase, as well as fibrosis, without normalization of blood pressure (BP). In contrast, administration of EXP3174 normalized BP and attenuated fibrosis but did not modify LOX, CCL and CTGF. In TGF- β_1 -stimulated fibroblasts, EXP3179 inhibited CTGF and LOX expression and activity with lower IC₅₀ values than EXP3174. Our results indicate that, despite a lower antihypertensive effect, EXP3179 shows higher anti-fibrotic efficacy than EXP3174, likely through its ability to prevent the excess of LOX and CCL. It is suggested that the anti-fibrotic effect of EXP3179 may be partially mediated by the blockade of CTGF-induced LOX in fibroblasts.

Myocardial fibrosis is involved in the development of left ventricular (LV) dysfunction and clinically overt heart failure (HF) in hypertensive patients^{1–3}. Diverse experimental studies indicate that collagen-dependent LV chamber stiffness is influenced not only by the amount of collagen fibers but also by the degree of collagen cross-linking (CCL) within the fibers, a process whereby collagen fibrils are covalently linked to one another by the enzyme lysyl oxidase (LOX), providing stiffness and resistance to degradation of the resulting fibers^{4–7}. Of notice, recent clinical studies point to an excess of CCL as a major determinant of LV dysfunction and clinical outcomes in patients with HF of hypertensive etiology^{1,8–10}. In this context, the reduction of myocardial LOX expression and CCL has been demonstrated to be associated with diminution of LV chamber stiffness in hypertensive patients with HF².

The angiotensin II type 1 receptor antagonist losartan is a prodrug metabolized by the cytochrome-P450 pathway in the liver producing two metabolites: EXP3174 and EXP3179. EXP3174 is the final metabolite and the pharmacological blocker of the AT₁ receptor by which losartan exerts its antihypertensive actions¹¹. EXP3179 is an intermediate metabolite that has no AT₁ receptor blocking properties and mediates a variety of AT₁ receptor-independent, non-hemodynamic actions^{12–15}. It is known that losartan reduces LV stiffness in hypertensive patients, an effect associated with the reduction of the content of myocardial collagen fibers¹⁶. However, there is no information on the effects of this drug on myocardial LOX and CCL in hypertension. Therefore, we have investigated the effects of EXP3179 and EXP3174 on fibrosis, and LOX and CCL in the myocardium of rats with arterial hypertension induced by the NG-nitro-L-arginine methyl ester (L-NAME), an experimental model characterized by myocardial transforming growth factor- β_1 (TGF- β_1) overexpression and fibrosis^{17–19}. In addition, we aimed to explore whether losartan metabolites influence LOX expression and activity in fibroblasts stimulated with TGF- β_1 .

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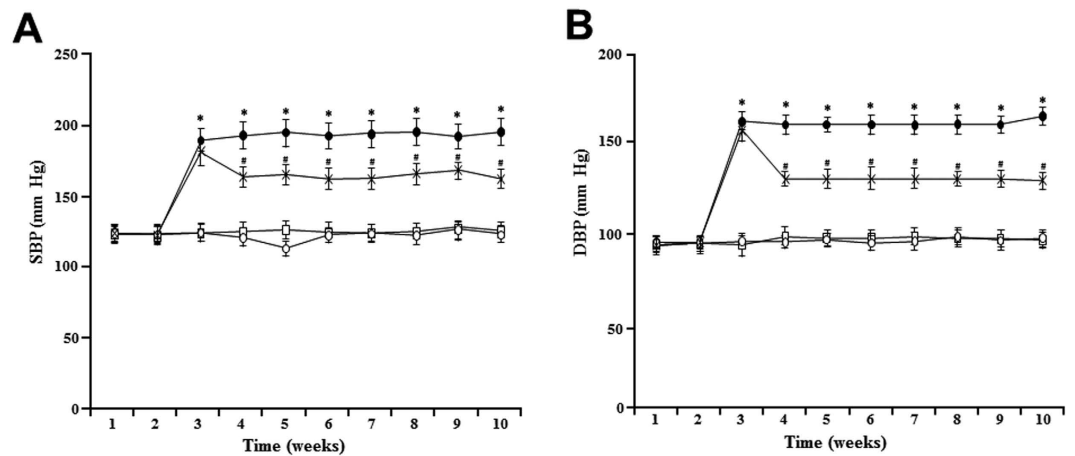


Figure 1. Time-course changes in systolic blood pressure (SBP) (panel A) and diastolic blood pressure (DBP) (panel B) in normotensive controls rats (□), L-NAME-treated rats (●), L-NAME + EXP3179-treated rats (x) and L-NAME + EXP3174-treated rats (○). Data points represent mean \pm SEM (n = 5). *P < 0.01 vs Control and L-NAME + EXP3174, #P < 0.05 vs L-NAME and Control.

Variables	Vehicle	L-NAME		
		+Vehicle	+EXP3179	+EXP3174
LV morphology				
RWT	0.42 \pm 0.01	0.53 \pm 0.02**	0.49 \pm 0.02*	0.52 \pm 0.01**
LVMI, mg/g	1.32 \pm 0.05	1.82 \pm 0.06**	1.45 \pm 0.02††	1.68 \pm 0.02**†
LV systolic function				
EF, %	72.3 \pm 0.83	63.1 \pm 3.35*	75.6 \pm 2.35†	73.5 \pm 3.28†
FS, %	43.7 \pm 0.45	35.1 \pm 2.34*	45.7 \pm 2.14†	44.1 \pm 2.70†
LV diastolic function				
E, cm/s	81.1 \pm 5.19	69.4 \pm 3.52*	82.8 \pm 2.03†	83.1 \pm 4.25†
A, cm/s	58.2 \pm 3.51	60.6 \pm 1.13	62.2 \pm 3.02	58.4 \pm 1.53
E/A	1.40 \pm 0.06	1.19 \pm 0.03*	1.35 \pm 0.07†	1.43 \pm 0.08†

Table 1. Echocardiographic Parameters. Data are expressed as mean \pm SEM. LV means left ventricle; RWT, relative wall thickness; LVMI, left ventricular mass index; EF, ejection fraction; FS, fractional shortening; E, peak velocity of early transmitral inflow; A, peak velocity of late transmitral inflow. *P < 0.05 vs Vehicle; †P < 0.05 vs L-NAME + Vehicle; **P < 0.01 vs Vehicle; ††P < 0.01 vs L-NAME + Vehicle.

Results

In Vivo Findings. *Effects of Losartan Metabolites on Blood Pressure (BP).* From the third week until the end of treatment, systolic and diastolic blood pressure (SBP and DBP) were elevated in L-NAME rats compared to control normotensive rats (Fig. 1). L-NAME + EXP3179 rats exhibited reduced SBP and DBP as compared with L-NAME rats, although BP values remained significantly increased as compared to control normotensive rats (Fig. 1A,B). On the other hand, L-NAME + EXP3174 rats showed SBP and DBP values similar to control normotensive rats throughout the entire 10-week treatment period (Fig. 1A,B). Treatment with either EXP3179 or EXP3174 in the absence of L-NAME did not influence either SBP or DBP (data not shown).

Effects of losartan metabolites on LV morphology and function. As shown in Table 1, compared to control normotensive rats, L-NAME rats exhibited LV hypertrophy, as indicated by the increased relative wall thickness (RWT) and LV mass index (LVMI). The LVMI was normalized in L-NAME + EXP3179 rats but remained increased in L-NAME + EXP3174 rats (Table 1).

In addition, Table 1 shows that L-NAME rats exhibited reduced LV systolic (e.g., reduced ejection fraction and fractional shortening) and diastolic (e.g., reduced E/A ratio) function compared to control normotensive rats. Co-treatment of L-NAME rats with either EXP3179 or EXP3174 prevented these alterations (Table 1). None of the parameters assessing LV morphology and function were modified in rats treated with either metabolite alone (data not shown).

Effects of Losartan Metabolites on Myocardial TGF- β , and CTGF Expression. Compared to control normotensive rats, L-NAME rats exhibited increased expression of myocardial TGF- β mRNA, which was fully prevented in L-NAME + EXP3179 and in L-NAME + EXP3174 rats (Fig. 2A). In addition, L-NAME rats showed increased expression of CTGF mRNA (Fig. 2B) and protein (Fig. 2C) compared to control normotensive rats. Co-treatment

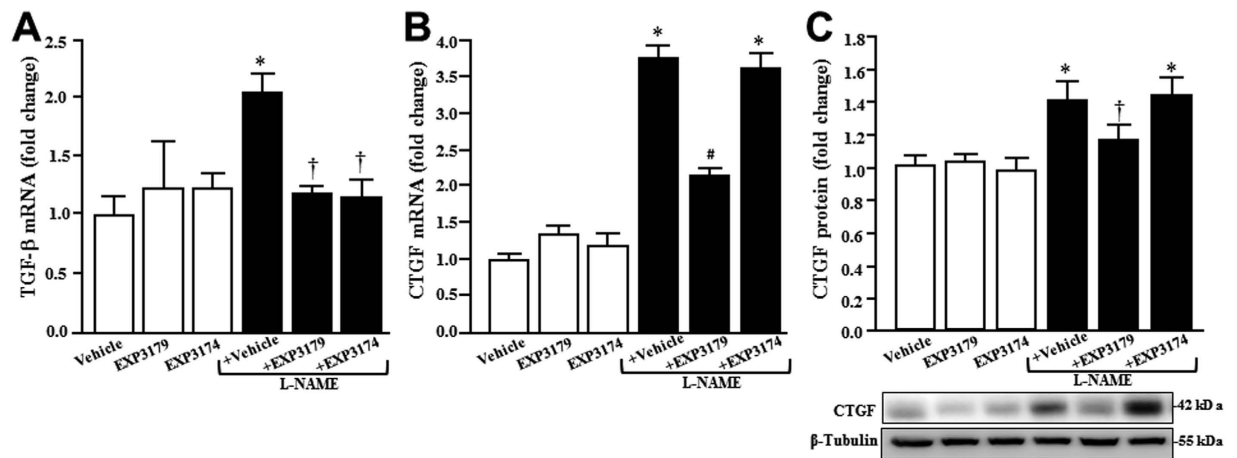


Figure 2. Histograms represent the expression of cardiac TGF- β_1 (panel A) and CTGF (panel B) mRNA and CTGF protein (panel C) in the myocardium of Vehicle-, EXP3179-, EXP3174-, L-NAME + Vehicle-, L-NAME + EXP3179- and L-NAME + EXP3174-treated rats. Representative Western blot autoradiograms for CTGF are presented at the bottom of panel C. Bars represent mean + SEM (n = 10). *P < 0.05 vs Vehicle, EXP3179 and EXP3174, †P < 0.05 vs L-NAME + Vehicle, #P < 0.05 vs Vehicle and L-NAME + Vehicle.

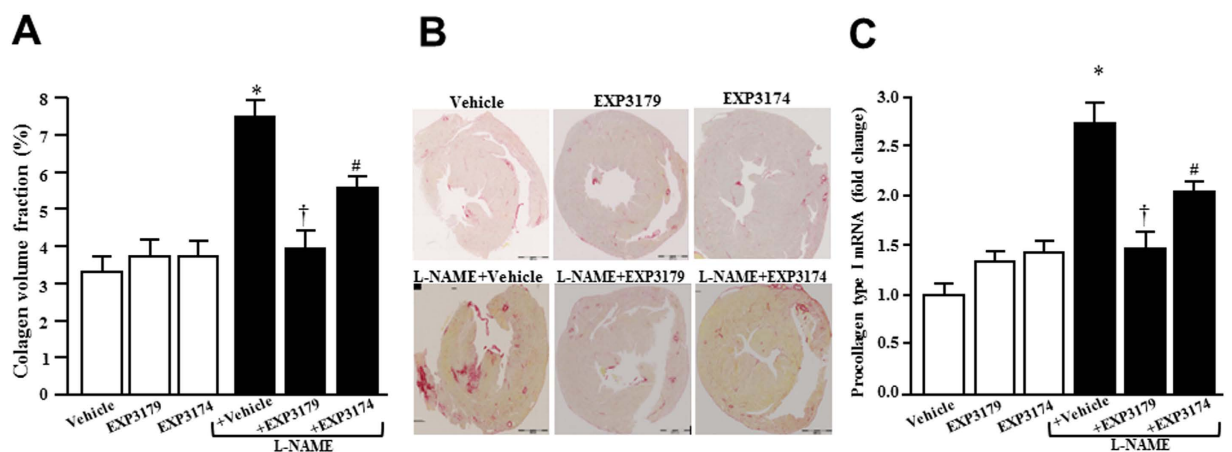


Figure 3. Histograms represent the percentage of collagen volume fraction (panel A) in the myocardium of Vehicle-, EXP3179-, EXP3174-, L-NAME + Vehicle-, L-NAME + EXP3179- and L-NAME + EXP3174-treated rats. Representative images of myocardial tissue from one rat of each group are shown in panel B. Sections were stained with picosirius red and collagen fibers were identified in red. Expression of procollagen type I mRNA is shown in panel C. Bars represent mean + SEM (n = 10). *P < 0.05 vs Vehicle, EXP3179 and EXP3174, †P < 0.05 vs L-NAME + Vehicle, #P < 0.05 vs Vehicle and L-NAME + Vehicle.

of L-NAME rats with EXP3179, but not with EXP3174, significantly decreased CTGF mRNA and protein expression induced by L-NAME (Fig. 2B,C, respectively). Treatment with either EXP3179 or EXP3174 in the absence of L-NAME did not exert any effect on these parameters (Fig. 2).

Effects of Losartan Metabolites on Myocardial Collagen Synthesis and Accumulation. Collagen volume fraction (CVF) was increased in L-NAME rats compared to control normotensive rats, being normal in L-NAME + EXP3179 rats but still abnormally increased in L-NAME + EXP3174 rats (Fig. 3A,B). Compared to control normotensive rats, L-NAME rats exhibited increased procollagen type I expression in cardiac tissue, which was fully prevented in L-NAME + EXP3179 rats and only partially prevented in L-NAME + EXP3174 rats (Fig. 3C). None of these parameters were modified in rats treated with either EXP3179 or EXP3174 in the absence of L-NAME (Fig. 3).

In addition, the myocardium of L-NAME rats exhibited increased LOX mRNA (Fig. 4A) and protein (Fig. 4B) expression, as well as an excess of CCL (Fig. 4C). These alterations were fully prevented in L-NAME + EXP3179 rats but not in L-NAME + EXP3174 rats. Treatment with either EXP3179 or EXP3174 in the absence of L-NAME did not exert any effect on these parameters (Fig. 4).

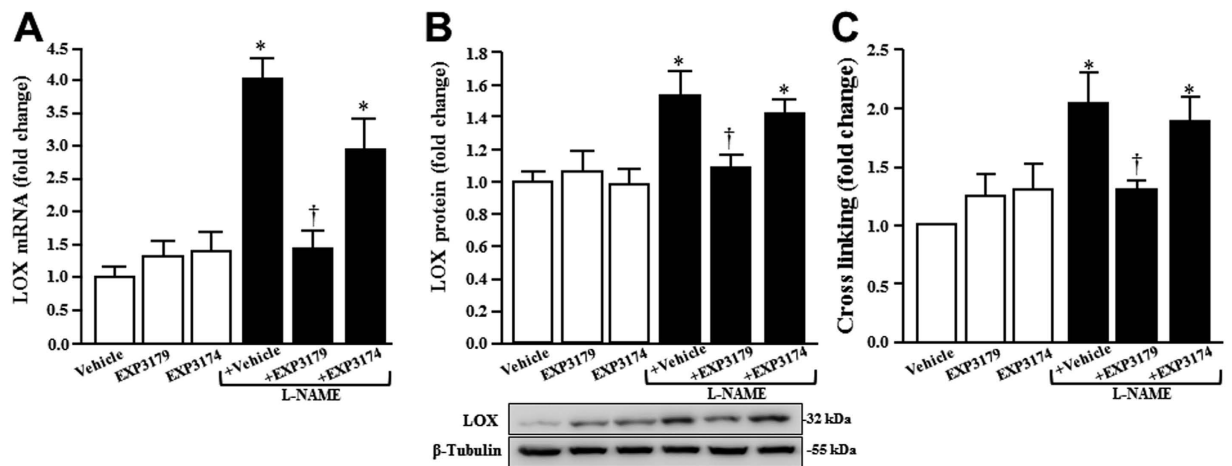


Figure 4. Histograms represent the fold change in the expression of LOX mRNA (panel A), LOX protein (panel B) and collagen cross-linking (panel C) in the myocardium of Vehicle-, EXP3179-, EXP3174-, L-NAME + Vehicle-, L-NAME + EXP3179- and L-NAME + EXP3174-treated rats. Representative Western blot autoradiograms for LOX protein expression are presented at the bottom of panel B. Bars represent mean + SEM (n = 10). *P < 0.05 vs Vehicle, EXP3179 and EXP3174, †P < 0.05 vs L-NAME + Vehicle.

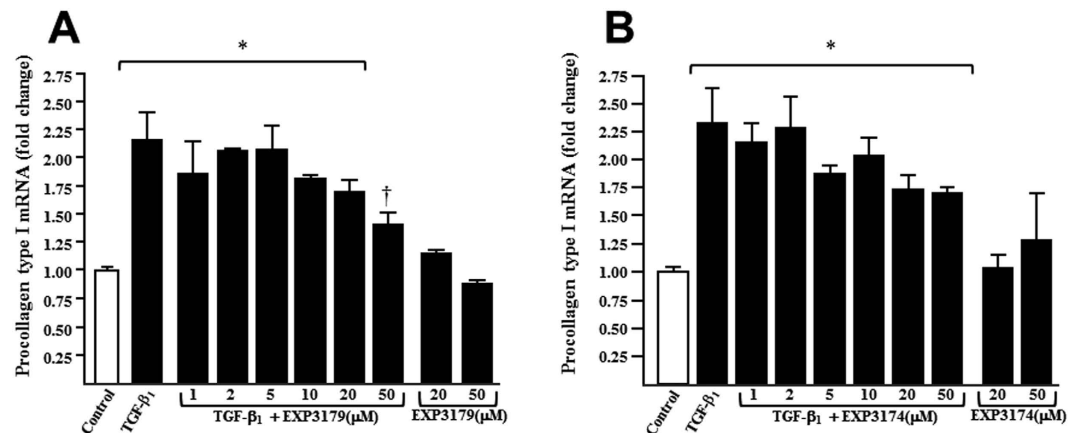


Figure 5. Histograms represent the fold change in procollagen type I mRNA expression in HDFa fibroblasts stimulated without and with 10^{-4} μ g/mL TGF- β_1 for 24 hours, in the absence or the presence of 1, 2, 5, 10, 20 and 50 μ M of EXP3179 (panel A) and EXP3174 (panel B), or incubated with the two compounds alone at 20 and 50 μ M, as compared with controls cells. Bars represent mean + SEM (n = 5 to 8 experiments). *P < 0.05 vs Control, †P < 0.05 vs TGF- β_1 .

In Vitro Findings. *Effects of Losartan Metabolites on Procollagen Type I Expression, and LOX Expression and Activity in TGF- β_1 -stimulated adult human dermal fibroblasts (HDFa).* As it has been previously published for cardiac fibroblast²⁰, incubation of HDFa with TGF- β_1 for 24 h induced a dose-dependent increase in both procollagen type I mRNA (see Supplementary Fig. S1A) and LOX (see Supplementary Fig. S1B) expression. TGF- β_1 10^{-4} μ g/mL was selected as the lowest concentration with submaximal effects on these parameters (see Supplementary Fig. S1).

HDFa fibroblasts were incubated with 10^{-4} μ g/mL TGF- β_1 in the presence of EXP3179 and EXP3174 at increasing concentrations. TGF- β_1 -induced procollagen type I expression was significantly inhibited by EXP3179 (Fig. 5A) and it tended to be reduced by EXP3174 (Fig. 5B).

In addition, EXP3179 inhibited TGF- β_1 effects on LOX mRNA (Fig. 6A) and protein (Fig. 6B) expression, with a clear tendency to inhibit extracellular LOX activity (Fig. 6C). Moreover, EXP3174 inhibited TGF- β_1 -induced LOX mRNA expression (Fig. 6D) and tended to reduce TGF- β_1 -induced LOX protein (Fig. 6E) and activity (Fig. 6F) only at high doses.

For all LOX parameters, EXP3179 was identified as a more potent inhibitor as compared to EXP3174 (Fig. 6G–I), with lower IC₅₀ values than EXP3174 (LOX mRNA: 1.6 ± 0.1 vs 29.4 ± 1.5 μ M, P < 0.001; LOX protein: 8.9 ± 1.0 vs 38.5 ± 5.0 μ M, P < 0.05; LOX activity: 7.4 ± 0.3 vs 37.3 ± 2.9 μ M, P < 0.05).

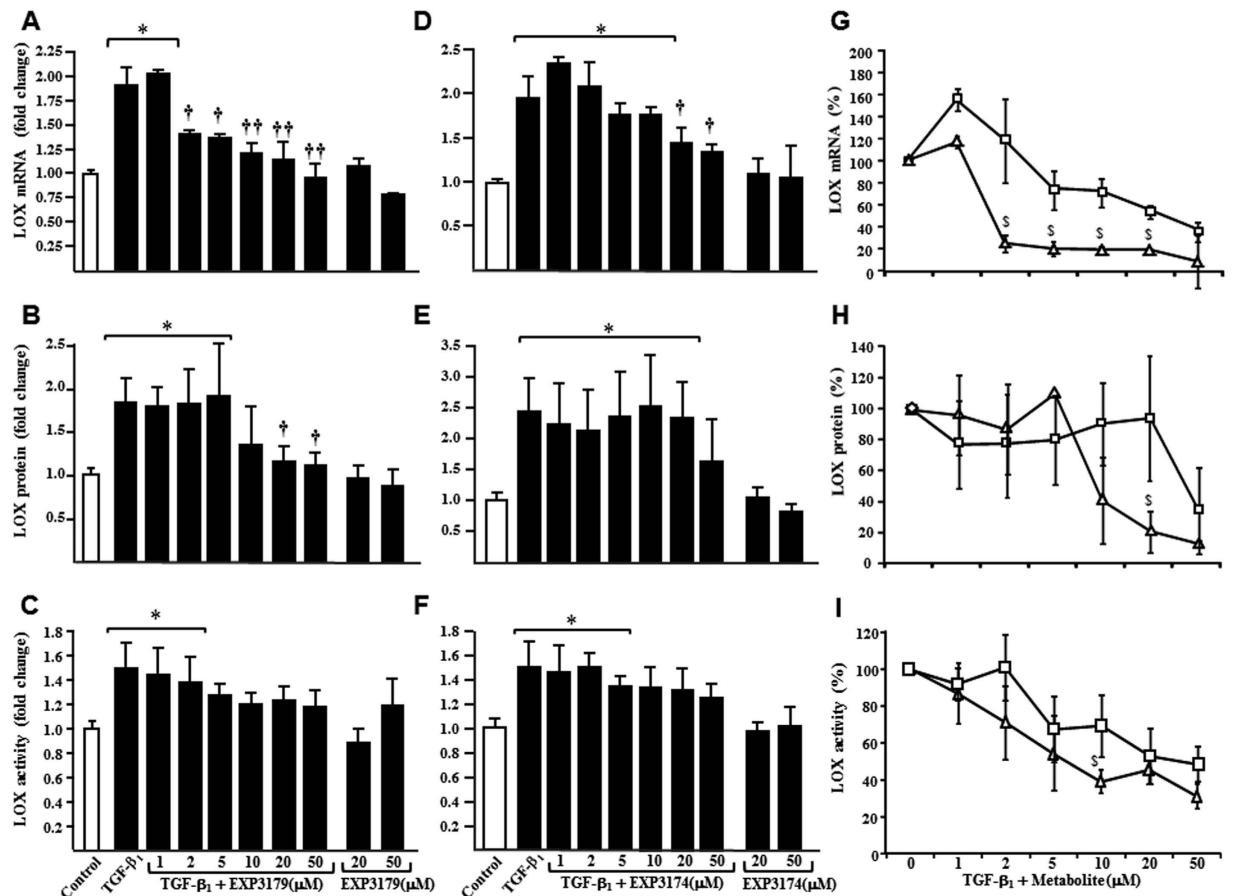


Figure 6. Histograms depict the fold change in LOX mRNA, protein and activity in HDFa fibroblasts stimulated without and with 10^{-4} $\mu\text{g}/\text{mL}$ TGF- β_1 for 24 hours, in the absence or the presence of 1, 2, 5, 10 and 50 μM of EXP3179 (panels A–C) and EXP3174 (panels D–F), or incubated with the two compounds alone at 20 and 50 μM , as compared to control cells. Line graphs show percentage of inhibition in LOX mRNA (panel G), protein (panel H), and activity (panel I), in HDFa stimulated with 10^{-4} $\mu\text{g}/\text{mL}$ TGF- β_1 in combination with EXP3174 (\square) or EXP3179 (Δ) at the above mentioned concentrations, compared to cells incubated with TGF- β_1 alone. Bars represent mean \pm SEM ($n = 5$ to 8 experiments) and curves show means \pm SEM ($n = 5$ to 8). * $P < 0.05$ vs Control, $^{\dagger}P < 0.05$ vs TGF- β_1 , $^{**}P < 0.01$ vs TGF- β_1 and $^{\$}P < 0.05$ vs EXP3174.

Involvement of Intracellular Pathways on EXP3179-mediated Inhibition of LOX Expression. We investigated whether classical intracellular pathways involved in the actions described for EXP3179 in other experimental settings^{12–15} were also involved in its inhibitory effects on TGF- β_1 -induced LOX upregulation in HDFa fibroblasts. However, none of the compounds tested (i.e. the inhibitors of PPAR- γ [G3335] and PI3K [LY294002], and the activators of PKC [PMA] and COX [LPS]) were able to counteract the actions of EXP3179 on LOX mRNA expression (see Supplementary Fig. S2).

In addition, we analysed in HDFa fibroblasts the involvement of diverse fibrosis-related genes in the EXP3179-induced LOX inhibition, by using the RT2 ProfilerTM PCR Array Human Fibrosis. As shown in table S1, 84 genes were examined, confirming the TGF- β_1 -induced increment in LOX expression and its inhibition in the presence of EXP3179, both exceeding a 1.5 fold change. Therefore, this threshold was chosen to select those genes overexpressed by TGF- β_1 that were inhibited by EXP3179. According to the array results and following the proposed criteria, CTGF and thrombospondin-1 (THBS1) were selected as genes that were potentially involved in the anti-fibrotic actions of EXP3179 (Supplemental Table S1). The inhibitory actions of EXP3179 on these genes were confirmed by real-time RT-PCR (data not shown). Therefore, CTGF and THBS1 genes were selected for further analyses.

By silencing CTGF expression with siRNA in HDFa, TGF- β_1 -induced LOX expression was inhibited. On the contrary, siRNA-mediated THBS1 inhibition did not exert any influence on this pathway (Supplementary Fig. S3A). In addition, we observed that, as in the case of LOX, EXP3179 inhibited CTGF mRNA expression in a dose-dependent manner (see Supplementary Fig. S3B).

Discussion

The main findings of this study are the following: 1) Chronic inhibition of NO synthesis with L-NAME in Wistar rats resulted in a hypertensive model of myocardial fibrosis with increased expression of CTGF and LOX, as well

as enhanced CCL; 2) Administration of EXP3179 in L-NAME rats exerted a partial anti-hypertensive effect, whereas EXP3174 fully prevented the increase in BP; 3) Whereas the administration of EXP3179 in L-NAME rats fully prevented myocardial CTGF and LOX overexpression, and excessive CCL and fibrosis, EXP3174 administration was not able to prevent LOX overexpression and CCL, and only partially reduced myocardial fibrosis; and 4) EXP3179 inhibited the TGF- β_1 -induced upregulation of LOX expression and activity in fibroblasts, probably through CTGF regulation, in a more effective manner than EXP3174.

The evidence supports that L-NAME-induced hypertension is a well-established experimental model, representative of the left ventricular remodeling, and specifically of the myocardial fibrosis, occurring in hypertensive heart disease^{17–19,21–25}. In addition, Tsukamoto *et al.* further characterized this model demonstrating that the lack of NO is associated with impairment of LV systolic and diastolic function²². In this regard, we confirm these findings and expand the characterization of myocardial fibrosis in this model with novel data demonstrating that reduced NO bioavailability is associated with increased myocardial LOX expression and enhanced cross-linking of collagen fibrils.

Although several clinical^{16,26,27} and experimental studies^{28–30} have demonstrated that losartan prevents and/or regresses the myocardial fibrosis associated with arterial hypertension, the role of losartan metabolites in this anti-fibrotic effect has not been characterized. In this regard, we report here that administration of EXP3179 completely prevented the excess of CTGF, LOX and CCL, and fully abrogated myocardial fibrosis, yet without normalization of BP. In contrast, we found that although administration of EXP3174 normalized BP, it failed to prevent myocardial CTGF and LOX overexpression, as well as the excess of CCL, and only partially reduced myocardial fibrosis. Of notice, both treatments prevented LV systolic and diastolic dysfunction. Therefore, we may speculate that EXP3179 may improve LV function by inhibiting CTGF-dependent pro-fibrotic mechanisms in cardiac fibroblasts, with a mild anti-hypertensive effect, whereas EXP3174 may improve LV function through normalization of BP with a concomitant, mild inhibitory effect on myocardial fibrosis.

In order to further evaluate the potential molecular mechanisms involved in the differential anti-fibrotic effects of losartan metabolites, *in vitro* experiments were performed. In accordance with previous studies^{20,31}, we observed an upregulation of procollagen type I and LOX expression and activity in fibroblasts stimulated with TGF- β_1 . In addition, we observed that CTGF mediates TGF- β_1 -induced LOX expression, confirming previous studies in which such an association was suggested³². Moreover, we report that EXP3179 showed a higher efficacy than EXP3174 in reducing LOX expression in HDFa, with significant effects at doses similar to those present in blood from patients chronically treated with losartan ($\sim 2\mu\text{M}$)³³. In this regard, examination of a large number of genes in the fibrosis pathway and the *in vitro* experiments with specific siRNAs revealed that EXP3179 effects on LOX are likely mediated through downregulation of CTGF. Our results provide mechanistic support to previous findings demonstrating that losartan is able to interfere with the profibrotic activity of TGF- β_1 , including downregulation of CTGF^{34–36}. In this regard, since CTGF may be a critical mediator of the EXP3179 actions on LOX expression, evaluation of different intracellular pathways participating in the regulation of CTGF (e.g., the c-Jun N-terminal kinase [JNK] pathway)^{37,38} could be considered for future studies. This is of particular interest taking into account that we observed that rats co-treated with L-NAME and EXP3174 exhibited CTGF overexpression despite TGF- β_1 being normalized, with a mild anti-fibrotic effect. In this regard, the TGF- β_1 -independent upregulation of CTGF and collagen synthesis induced by angiotensin II has been reported in different *in vivo* models of renal damage³⁹, and in atrial fibrillation⁴⁰. In addition, other mechanisms besides TGF- β_1 , and related to the renin-angiotensin system, hemodynamic stress⁴¹ or inflammation/oxidative stress¹⁷, have been reported as inductors of myocardial fibrosis in L-NAME-treated rats. On the other hand, the mild antifibrotic effect shown by EXP3174 may be due to abrogation of chronic hemodynamic stress, independently of CTGF. This notion is supported by previous studies demonstrating that treatment with losartan or hydrochlorothiazide showed similar anti-fibrotic and anti-hypertensive effects in the myocardium of SHR rats receiving high salt diet, being losartan more effective in decreasing CTGF expression⁴².

Some limitations of the present study must be acknowledged. First, a losartan group was not included in the study. Nevertheless, the aim of the study was to examine the effects of both metabolites on myocardial fibrosis present in L-NAME rats, in order to determine their individual anti-fibrotic actions on the hypertensive myocardium. Second, few studies have applied EXP3179 as an individual potential drug and therefore a dose-dependent pharmacological profile of EXP3179 has not been established yet. Third, further experiments are needed to analyse the myocardial distribution of extracellular matrix/profibrotic proteins in the L-NAME-induced hypertension model. Fourth, in the *in vitro* study, the potential contribution of the remaining differentially expressed genes in the array was not analysed in depth. In addition, further studies are required to confirm these findings in human cardiac fibroblasts. Finally, it is unknown whether the anti-fibrotic effects of EXP3179 were solely due to prevention of collagen synthesis and deposition or if the metabolite also influences the enzymes that control collagen degradation.

We conclude that despite a lower hypertensive efficacy, the losartan metabolite EXP3179 is more effective improving myocardial fibrosis than EXP3174 in L-NAME rats. In particular, we found that EXP3179 is more efficient than EXP3174 downregulating LOX expression, with these effects resulting in a normalization of CCL. Of interest, these molecular and anti-fibrotic effects of EXP3179 in L-NAME rats may be mediated by the inhibition of the TGF- β_1 -CTGF pathway that is activated in the myocardium of these rats.

Methods

For detailed description, see Methods in the online Data Supplement.

In Vivo Procedure. Forty five 10-week-old male Wistar rats were treated for 10 weeks, once-daily, with 30 mg/kg of L-NAME by oral administration, a dose sufficient to induce arterial hypertension⁴³ and 45 untreated rats were considered as controls. Fifteen animals in each group were co-treated, once-daily, with either vehicle,

EXP3179 or EXP3174 by oral administration. EXP3174 was administered at 5 mg/kg/day, a dose resulting in circulating metabolite levels similar to those found in patients chronically treated with losartan^{33,44}. EXP3179 was administered at the same dose. Blood pressure (BP) was assessed in five animals from each group by radiotelemetry monitoring.

Echocardiographic Studies. Echocardiography was performed using a Vevo 770 ultrasound system. The heart rate (HR) of the animals was recorded immediately before the echocardiographic study.

Collagen Cross-linking Analysis. The evaluation of the degree of CCL was performed using Fast Green-Sirius Red and Sircol-based colorimetric assays in myocardial tissue.

Assessment of Collagen Volume Fraction. CVF was determined in heart sections of rats as a percentage of total myocardial area occupied by collagen tissue.

In Vitro Procedure. After carrying out dose-response curves to human recombinant TGF- β_1 (R α D systems), adult human dermal fibroblasts (HDFa line; GIBCO) were incubated for 24 hours with or without 10^{-4} μ g/mL, in the absence or presence of the metabolites EXP3174 and EXP3179 (provided by Merck & Co, Inc.) at a range of concentrations. In addition, HDFa cells were co-incubated with EXP3179 in the absence or presence of the following compounds: the inhibitors of PPAR- γ (G3335) and phosphatidylinositol 3-kinase (PI3K) pathway (LY294002) and the activators of protein kinase-C (PKC) (Phorbol 12-myristate 13-acetate [PMA]) and cyclo-oxygenase-2-pathway (lipopolysaccharide [LPS]).

Human fibrosis array. Samples from cells incubated in control conditions or with TGF- β_1 , in the absence or presence of EXP3179 (20 μ M) were examined by using the Human Fibrosis RT2 Profiler PCR Array (SABiosciences Corp.) consisting of a panel of 84 key genes involved in fibrosis.

Quantitative Real-time PCR and Gene Silencing. Gene expression was analysed by quantitative real-time PCR by using specific TaqMan fluorescent probes. Expression of connective tissue growth factor (CTGF) and thrombospondin 1 (THBS1) was silenced by small interfering RNAs in HDFa.

Assessment of Protein Expression and Evaluation of LOX Activity. The expression of several proteins was analysed by Western Blot. LOX activity was assessed by a commercially available fluorimetric assay.

Statistical Analysis. Variables are expressed as means \pm SEM. Differences among different conditions were tested by one-way ANOVA followed by the Fisher's least significant difference method for post-hoc comparisons once normality was checked (Shapiro-Wilks test); otherwise, the nonparametric Kruskal-Wallis test followed by a Mann-Whitney U test (adjusting the α -level by Bonferroni inequality) was used. Statistical significance was defined as two-sided $P < 0.05$. The analyses were performed using the programs SPSS (15.0 version) and STATA (12.1 version).

Ethical standards. All authors in this work gave their informed consent prior to their participation in the study. The manuscript does not contain clinical studies or patient data. The research conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996), and was approved by the Ethical Committee for Animal Experimentation of the University of Navarra (036/08).

References

- Zile, M. R. *et al.* Myocardial stiffness in patients with heart failure and a preserved ejection fraction: contributions of collagen and titin. *Circulation* **131**, 1247–1259 (2015).
- López, B., Querejeta, R., González, A., Beaumont, J., Larman, M. & Díez, J. Impact of treatment on myocardial lysyl oxidase expression and collagen cross-linking in patients with heart failure. *Hypertension* **53**, 236–242 (2009).
- Dai, Z., Aoki, T., Fukumoto, Y. & Shimokawa, H. Coronary perivascular fibrosis is associated with impairment of coronary blood flow in patients with non-ischemic heart failure. *J Cardiol.* **60**, 416–421 (2012).
- Norton, G. R., Tsotetsi, J., Trifunovic, B., Hartford, C., Candy, G. P. & Woodiwiss, A. J. Myocardial stiffness is attributed to alterations in cross-linked collagen rather than total collagen or phenotypes in spontaneously hypertensive rats. *Circulation* **96**, 1991–1998 (1997).
- Badenhorst, D. *et al.* Cross-linking influences the impact of quantitative changes in myocardial collagen on cardiac stiffness and remodelling in hypertension in rats. *Cardiovasc Res.* **57**, 632–641 (2003).
- Shoulders, M. D. & Raines, R. T. Collagen structure and stability. *Annu Rev Biochem.* **78**, 929–958 (2009).
- Brower, G. L. *et al.* The relationship between myocardial extracellular matrix remodeling and ventricular function. *Eur J Cardiothorac Surg.* **30**, 604–610 (2006).
- Kasner, M. *et al.* Diastolic tissue Doppler indexes correlate with the degree of collagen expression and cross-linking in heart failure and normal ejection fraction. *J Am Coll Cardiol* **57**, 977–985 (2011).
- López, B., Querejeta, R., González, A., Larman, M. & Díez, J. Collagen cross-linking but not collagen amount associates with elevated filling pressures in hypertensive patients with stage C heart failure: potential role of lysyl oxidase. *Hypertension* **60**, 677–683 (2012).
- López, B. *et al.* Myocardial collagen cross-linking is associated with hospitalization for heart failure in patients with hypertensive heart failure. *J Am Col Cardiol* **67**, 251–260 (2016).
- Lo, M. W., Goldberg, M. R., McCreary, J. B., Lu, H., Furtek, C. I. & Bjornsson, T. D. Pharmacokinetics of losartan, an angiotensin II receptor antagonist, and its active metabolite EXP3174 in humans. *Clin Pharmacol Ther* **58**, 641–649 (1995).
- Krämer, C. *et al.* Angiotensin II receptor-independent antiinflammatory and antiaggregatory properties of losartan: role of the active metabolite EXP3179. *Circ Res* **90**, 770–776 (2002).
- Watanabe, T., Suzuki, J., Yamawaki, H., Sharma, V. K., Sheu, S. S. & Berk, B. C. Losartan metabolite EXP3179 activates Akt and endothelial nitric oxide synthase via vascular endothelial growth factor receptor-2 in endothelial cells: angiotensin II type 1 receptor-independent effects of EXP3179. *Circulation* **112**, 1798–1805 (2005).

14. Fortuño, A. *et al.* Losartan metabolite EXP3179 blocks NADPH oxidase-mediated superoxide production by inhibiting protein kinase C. Potential clinical implications in hypertension. *Hypertension* **54**, 744–750 (2009).
15. Schupp, M. *et al.* Regulation of peroxisome proliferator-activated receptor gamma activity by losartan metabolites. *Hypertension* **47**, 586–589 (2006).
16. Díez, J., Querejeta, R., López, B., González, A., Larman, M. & Martínez Ubago, J. L. Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients. *Circulation* **105**, 2512–2517 (2002).
17. Ndisang, J. F., Chibbar, R. & Lane, N. Heme oxygenase suppresses markers of heart failure and ameliorates cardiomyopathy in L-NAME-induced hypertension. *Eur J Pharmacol* **734**, 23–34 (2014).
18. Zambrano, S. *et al.* L-Carnitine protects against arterial hypertension-related cardiac fibrosis through modulation of PPAR- γ expression. *Biochem Pharmacol* **85**, 937–944 (2013).
19. Silambarasan, T., Manivannan, J., Krishna Priya, M., Suganya, N., Chatterjee, S. & Raja, B. Sinapic acid prevents hypertension and cardiovascular remodeling in pharmacological model of nitric oxide inhibited rats. *PLoS One* **9**, e115682 (2014).
20. Voloshenyuk, T. G., Landesman, E. S., Khouctorova, E., Hart, A. D. & Gardner, J. D. Induction of cardiac fibroblast lysyl oxidase by TGF- β 1 requires PI3K/Akt, Smad3, and MAPK signaling. *Cytokine* **55**, 90–97 (2011).
21. Bunbupha, S., Prachaney, P., Kukongviriyapan, U., Kukongviriyapan, V., Welbat, J. U. & Pakdeechote, P. Asiatic acid alleviates cardiovascular remodelling in rats with L-NAME-induced hypertension. *Clin Exp Pharmacol Physiol* **42**, 1189–1197 (2015).
22. Tsukamoto, O. *et al.* The antagonism of aldosterone receptor prevents the development of hypertensive heart failure induced by chronic inhibition of nitric oxide synthesis in rats. *Cardiovasc Drug Ther* **20**, 93–102 (2006).
23. Yamashita, T. *et al.* Apoptosis signal-regulating kinase-1 is involved in vascular endothelial and cardiac remodeling caused by nitric oxide deficiency. *Hypertension* **50**, 519–524 (2007).
24. Ferreira-Melo, S. E. *et al.* Sildenafil reduces cardiovascular remodeling associated with hypertensive cardiomyopathy in NOS inhibitor-treated rats. *Eur J Pharmacol* **542**, 141–147 (2006).
25. Tomita, H. *et al.* Early induction of transforming growth factor-beta via angiotensin II type 1 receptors contributes to cardiac fibrosis induced by long-term blockade of nitric oxide synthesis in rats. *Hypertension* **32**, 273–279 (1998).
26. Shimada, Y. J. *et al.* Effects of losartan on left ventricular hypertrophy and fibrosis in patients with nonobstructive hypertrophic cardiomyopathy. *J Am Coll Cardiol HF* **1**, 480–487 (2013).
27. López, B. *et al.* Usefulness of serum carboxy-terminal propeptide of procollagen type I in assessment of the cardioreparative ability of antihypertensive treatment in hypertensive patients. *Circulation* **104**, 286–291 (2001).
28. Varo, N. *et al.* Losartan inhibits the post-transcriptional synthesis of collagen type I and reverses left ventricular fibrosis in spontaneously hypertensive rats. *J Hypertens* **17**, 107–114 (1999).
29. Boffa, J. J., Lu, Y., Placier, S., Stefanski, A., Dussaule, J. C. & Chatziantoniou C. Regression of renal vascular and glomerular fibrosis: role of angiotensin II receptor antagonism and matrix metalloproteinases. *J Am Soc Nephrol* **14**, 1132–1144 (2003).
30. Varo, N., Iraburu, M. J., Varela, M., López, B., Etayo, J. C. & Díez, J. Chronic AT(1) blockade stimulates extracellular collagen type I degradation and reverses myocardial fibrosis in spontaneously hypertensive rats. *Hypertension* **35**, 1197–1202 (2000).
31. Roy, R., Polgar, P., Wang, Y., Goldstein, R. H., Taylor, L. & Kagan, H. M. Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF- β , IL-1 β , and prostaglandin E. *J Cell Biochem* **62**, 411–417 (1996).
32. Hong, H. H., Uzel, M. I., Duan, C., Sheff, M. C. & Trackman, P. C. Regulation of lysyl oxidase, collagen, and connective tissue growth factor by TGF- β 1 and detection in human gingiva. *Lab Invest* **79**, 1655–1667 (1999).
33. Kappert, K. *et al.* Chronic treatment with losartan results in sufficient serum levels of the metabolite EXP3179 for PPAR γ activation. *Hypertension* **54**, 738–743 (2009).
34. Cohn, R. D. *et al.* Angiotensin II type 1 receptor blockade attenuates TGF- β -induced failure of muscle regeneration in multiple myopathic states. *Nat Med* **13**, 204–210 (2007).
35. Geirsson, A. *et al.* Modulation of transforming growth factor- β signaling and extracellular matrix production in myxomatous mitral valves by angiotensin II receptor blockers. *Circulation* **126**, S189–197 (2012).
36. Friedberg, M. K. *et al.* Adverse biventricular remodeling in isolated right ventricular hypertension is mediated by increased transforming growth factor- β 1 signaling and is abrogated by angiotensin receptor blockade. *Am J Respir Cell Mol Biol* **49**, 1019–1028 (2013).
37. Shi, L., Chang, Y., Yang, Y., Zhang, Y., Yu, F. S. & Wu, X. Activation of JNK signalling mediates connective tissue growth factor expression and scar formation in corneal wound healing. *PLoS One* **7**, e32128 (2012).
38. Weng, C. M., Yu, C. C., Kuo, M. L., Chen, B. C. & Lin, C. H. Endothelin-1 induces connective tissue growth factor expression in human lung fibroblasts by ETAR-dependent JNK/AP-1 pathway. *Biochem Pharmacol* **88**, 402–411 (2014).
39. Yang, F., Chung, A. C., Huang, X. R. & Lan, H. Y. Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-beta-dependent and -independent Smad pathways: the role of Smad3. *Hypertension* **54**, 877–884 (2009).
40. Kiryu, M. *et al.* Angiotensin II-mediated up-regulation of connective tissue growth factor promotes atrial tissue fibrosis in the canine atrial fibrillation model. *Europace* **14**, 1206–1214 (2012).
41. Sadek, S. A., Rashed, L. A., Bassam, A. M. & Said, E. S. Effect of aliskiren, telmisartan and torsemide on cardiac dysfunction in l-nitro arginine methyl ester (l-NAME) induced hypertension in rats. *J Adv Res* **6**, 967–974 (2015).
42. Gröholm, T. *et al.* Cardioprotective effects of vasopeptidase inhibition vs. angiotensin type 1-receptor blockade in spontaneously hypertensive rats on a high salt diet. *Hypertens Res* **27**, 609–618 (2004).
43. Morán, A., de Urbina, A. V., Martín, M. L., Rodríguez-Barbero, A. & Román, L. S. Characterization of the contractile 5-hydroxytryptamine receptor in the autoperfused kidney of L-NAME hypertensive rats. *Eur J Pharmacol* **620**, 90–96 (2009).
44. Yan, Y. D. *et al.* The physicochemical properties, *in vitro* metabolism and pharmacokinetics of a novel ester prodrug of EXP3174. *Mol Pharm* **7**, 2132–2140 (2010).

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Author Contributions

J.L.M.-C., J.B. and G.S.J., co-designed the study, conducted animal studies and revised the manuscript critically for important intellectual content. M.U.M., B.L. and A.G., co-designed the study, performed experiments and revised the manuscript critically for important intellectual content. G.Z., A.F., S.R. and J.D., co-designed the study, wrote and edited the manuscript. All authors reviewed and approved the final manuscript.

Additional Information

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