



**HELMHOLTZ  
ZENTRUM FÜR  
INFEKTIONSFORSCHUNG**

**This is a pre- or post-print of an article published in  
Adam, O., Zimmer, C., Hanke, N., Hartmann, R.W., Klemmer,  
B., Böhm, M., Laufs, U.  
Inhibition of aldosterone synthase (CYP11B2) by  
torasemide prevents atrial fibrosis and atrial  
fibrillation in mice  
(2015) Journal of Molecular and Cellular Cardiology, 85,  
pp. 140-150.**

## Revision of JMCC8951

# **Inhibition of aldosterone synthase (CYP11B2) by torasemide prevents atrial fibrosis and atrial fibrillation in mice**

Oliver Adam MD, Christina Zimmer PhD\*, Nina Hanke PhD\*, Rolf W. Hartmann PhD\*, Birgit Klemmer PhD, Michael Böhm MD, Ulrich Laufs MD

Klinik für Innere Medizin III, Kardiologie, Angiologie und Internistische Intensivmedizin;  
Universitätsklinikum des Saarlandes, D-66421 Homburg/Saar, Germany

\* Helmholtz-Institute for Pharmaceutical Research Saarland & Pharmaceutical and Medicinal Chemistry, Saarland University

Adam: CYP11B2 inhibition and atrial fibrosis

Word count including title page, abstract, text, tables, figure legends, references:

### **Sources of Funding**

This study was supported by the Deutsche Forschungsgemeinschaft (DFG), the Universität des Saarlandes (HOMFOR) and the European Section of the Aldosterone Council (ESAC).

### **Disclosures**

All authors have no disclosures or conflict of interest.

### **Corresponding author:**

Oliver Adam, MD

Klinik für Innere Medizin III, Kardiologie, Angiologie und Internistische Intensivmedizin;  
Universitätsklinikum des Saarlandes

D-66421 Homburg/Saar, Germany

e-mail:oliver.adam@uks.eu

## **Abstract**

**Background:** Loop diuretics are used for fluid control in patients with heart failure. Furosemide and torasemide may exert differential effects on myocardial fibrosis.

**Objectives:** Here, we studied the effects of torasemide and furosemide on atrial fibrosis and remodeling during atrial fibrillation

**Methods and Results:** In primary neonatal cardiac fibroblasts, torasemide (50  $\mu$ M, 24 h) but not furosemide (50  $\mu$ M, 24 h) reduced the expression of connective tissue growth factor (CTGF; 65 $\pm$ 6%) and the pro-fibrotic miR-21 (44 $\pm$ 23%), as well as the expression of lysyl oxidase (LOX; 57 $\pm$ 8%), a regulator of collagen crosslinking. Mineralocorticoid receptor (MR) expression and activity were not altered. Torasemide but not furosemide inhibited human aldosterone synthase (CYP11B2) activity in transfected lung fibroblasts (V79MZ cells) by 75 $\pm$ 1.8%. The selective CYP11B2 inhibitor SL242 mimicked the torasemide effects. Mice with cardiac overexpression of Rac1 GTPase (RacET), which develop atrial fibrosis and spontaneous AF with aging, were treated long-term (8 months) with torasemide (10mg/kg/day), furosemide (40mg/kg/day) or vehicle. Treatment with torasemide but not furosemide prevented atrial fibrosis in RacET as well as the up-regulation of CTGF, LOX and miR-2, whereas MR expression and activity remained unaffected. These effects correlated with a reduced prevalence of atrial fibrillation (33% RacET+Tora vs. 80% RacET).

**Conclusion:** Torasemide but not furosemide inhibits CYP11B2 activity and reduces the expression of CTGF, LOX and miR-21. These effects are associated with prevention of atrial fibrosis and a reduced prevalence of atrial fibrillation in mice.

**Key Words:** atrial fibrillation, fibrosis, loop diuretics, torasemide

## **Abbreviations**

AF = atrial fibrillation

AngII = angiotensin II

CTGF = connective tissue growth factor

CY11B2= aldosterone synthase

LA = left atrium

LOX = lysyloxidase

miR-21= microRNA-21

MR = mineralocorticoid receptors

RacET = transgenic mice with cardiac overexpression of Rac1 GTPase

SR = sinus rhythm

WT = wild-type mice

## 1. Introduction

Loop diuretics, such as furosemide and torasemide, are used for symptomatic fluid control in patients with chronic heart failure (CHF) [1]. Both diuretics exert similar diuretic effects [2, 3] but their effect on clinical outcomes remains uncertain. Interestingly, previous studies have reported that torasemide – but not furosemide – decreased collagen volume fraction in the hearts of patients with chronic heart failure (CHF) [4], and early evidence suggests that torasemide treatment was associated with lower mortality compared to furosemide in the TORasemide In Chronic heart failure (TORIC) study [5].

Myocardial fibrosis is a hallmark of both left ventricular dysfunction and of atrial arrhythmogenic structural remodeling [6-8] which represents an important substrate for atrial fibrillation (AF) [9]. An important mediator of left ventricular and atrial fibrosis is the activation of mineralocorticoid receptors (MR) by aldosterone [10-15]. Interestingly, experimental and human studies have reported that torasemide, but not furosemide, may exert anti-aldosterone effects [16] [17, 18]. However, the underlying mechanisms and the consequences of these observations are only partially understood, especially in the context of atrial fibrillation.

Our previous studies found that the fibrosis in left atria of patients with AF is characterized by increased angiotensin II tissue concentrations as well as increased expression and activity of the small Rho-GTPase Rac1, increased expression of the connective tissue growth factor (CTGF) and increased expression of lysyl oxidase (LOX), a key enzyme of collagen crosslinking [19-23], as well as micro RNA-21 (miR-21), a regulator of ventricular [24] and atrial fibrosis [25]. This pro-fibrotic signalling pathway can be activated by aldosterone [26]. Based on these findings, the present study was undertaken to test the hypothesis of a potential differential effect of the loop-diuretics torasemide and furosemide on myocardial fibrosis and

atrial remodelling and to characterize the underlying signalling events. To this end, cultured primary cardiac cells and Rac1 transgenic mice were studied that spontaneously develop atrial fibrosis and atrial fibrillation with aging.

## **2. Materials and Methods**

### **2.1. Cell Isolation and Culture**

Cardiomyocytes and cardiac fibroblasts were isolated from the atria and the ventricles of 5 days old neonatal Sprague–Dawley rat hearts [27]. Purity of fibroblasts was confirmed by vimentin staining. After 48 h in culture, myocytes exhibited regular spontaneous contractions. The primary cells were used for experiments after 3–6 days of culture.

HL-1 cells, a cardiac muscle cell line from the AT-1 mouse atrial cardiomyocyte tumor lineage, were a gift from William C. Claycomb, Ph.D.; Professor of Biochemistry and Molecular Biology LSU Health Sciences Center; New Orleans) [50]. HL-1 cells maintain the ability to contract and retain differentiated cardiac morphological, biochemical, and electrophysiological properties [50].

Ventricular rat neonatal cells were used for the signalling experiments, the results were confirmed using pooled atrial neonatal cardiomyocytes and fibroblasts as well as HL-1 cells.

### **2.2. Animal Studies**

Mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the  $\alpha$ -myosin heavy chain (MHC) promoter (RacET) and wild type controls (WT; FVB-N-strain) promoter [28] were fed with normal chow (ssniff, Germany) or normal chow supplemented with 10 mg/kg/d of commercially available torasemide (Meda Pharma, Germany) or 40mg/kg/d furosemide (STADA, Germany) for 8 months. Heart rate, regularity and presence

of p-waves were documented by ECG (Picker, Schwarzer CU 12 system) under anaesthesia with ketamine (ketavet, 100mg/kgKG) and xylazine (rompun, 10mg/kgKG) ip. Transthoracic echocardiography (Vevo 770® high-resolution imaging system (Visual Sonics, Canada) with a center frequency of 30 MHz and a focal depth of 12.6mm) was performed in all mice under anaesthesia with ketamine (ketavet, 100mg/kgKG) and xylazine (rompun, 10mg/kgKG) ip; fractional shortening (FS), enddiastolic thickness of interventricular septum (IVSd) and the left posterior wall (LPWd) as well as the left ventricular enddiastolic diameter (LVDd) were taken. The characteristics of the mice are depicted in **Table 1**. None of the wildtype mice exhibited atrial arrhythmias [19, 20, 29].

The study was approved by the animal ethics committee of the Universität des Saarlandes and is in accord with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Pub. No. 85-23, revised 1996). The study was not funded by the pharmaceutical industry.

### **2.3. Reverse-transcriptase polymerase-chain reaction (RT-PCR)**

Total RNA isolation, reverse transcription, and competitive PCR was performed according to standard techniques. The sense, (5'- GATATCTTCAAAGAGAGG -3') and anti-sense (5'- TACTGTTCAGCTAATCACG -3') primers were used to amplify a CYP11B2 cDNA and (5'- TACTGTTCAGCTAATCACG -3') and anti-sense (5'- TACTGTTCAGCTAATCACGC -3') primers were used to amplify a MCR cDNA fragment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an external standard. Each PCR cycle consisted of denaturing at 94°C for 30 s, annealing at 53°C for 30 s, and elongation at 72 °C for 60 s. The linear exponential phases for CYP11B2, MCR and GAPDH PCR were 40 and 22 cycles, respectively. Equal amounts of corresponding CYP11B2, MR and GAPDH RT-PCR products were loaded on 1.5% agarose gels and optical densities of ethidium-bromide stained DNA bands were quantitated.

#### **2.4. Micro-RNA Isolation and RT-PCR**

MicroRNAs were isolated by a miRNA isolation kit (mirVana, Ambion). For real-time PCR, a target-specific stem loop structure and reverse transcription primers were used. After reverse transcription miR-21 expression was quantified with specific TaqMan hybridization probes (TaqMan miR-21 microRNA assay, Applied Biosystems). The small RNA molecule U6 small nuclear (*Rnu6-2*) was amplified as a control.

#### **2.5. Western Analysis**

Protein lysates were prepared as described [27]. Immunoblotting was performed using Anti-Rac1 (Rac1, Upstate, clone 23A8), CTGF (sc-14939, Santa Cruz; USA), LOX (ab31238, Abcam, UK) and GAPDH (ab8245, Abcam; UK). Immunodetection was accomplished using goat anti-rabbit or goat anti-mouse secondary antibody (1:4000 dilution, Sigma) and an enhanced chemiluminescence kit (Amersham) followed by densitometry.

#### **2.6. Rac1 GST-PAK Pull-Down Assay**

Pull-down assays were performed using agarose labelled PAK-1 fusion protein (Upstate, USA) as described (4).

#### **2.7. CYP11B2 Activity**

The untransfected V79MZ Chinese hamster cells (provided by Prof. Bernhardt, Saarland University) and the human CYP11B2 expressing V79MZ cell line were grown as monolayer culture in Dulbecco's modified Eagle medium (DMEM; c.c.pro, Oberdorla, Germany) supplemented with 5 % of fetal calf serum (FCS;Sigma), penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM) and sodium pyruvate (1 mM) at 37°C in 5 % CO<sub>2</sub> in air.



The cDNA of CYP11B2 was amplified from total RNA extracted from NCI-H295R cells (ATCC, Manassas, USA). Total RNA was isolated with the GenElute™ Total Mammalian Miniprep Kit (Sigma-Aldrich, Munich, Germany), following the manufacturer's instructions. cDNA synthesis was performed using Improm-II™ Reverse Transcriptase (Promega, Madison, USA) according to Promega's standard reverse transcription protocol. This procedure was followed by a polymerase chain reaction (PCR) to amplify the CYP11B2 gene with specific sense (5'-GCCACCATGGCACTCAGGGCAAAGGCAGAGG-3') and anti-sense (5'-CTAGTTAATCGCTCTGAAAGTGAGGAGGGGGGACG-3') primers. The purified PCR-product was cloned into MCS of pcDNA3.1/V5-His<sup>®</sup> TOPO<sup>®</sup> TA expression vector from invitrogen (Carlsbad, USA).

One day before transfection,  $2 \times 10^5$  untransfected V79MZ cells were seeded into 35 mm culture dishes (Nunc, Wiesbaden, Germany) and incubated overnight in complete growth medium. On the following day the approximately 70 % confluent culture was transfected with the generated plasmid using the liposomal transfecting reagent Roti<sup>®</sup>-Fect (Carl Roth, Karlsruhe, Germany) following the manufacturer's recommendations. Stably transfected clones carrying the Neomycin resistance marker were selected with 750 µg/ml G418 sulfate.

For determination of inhibitory effects on aldosterone synthase, V79MZ cells expressing human CYP11B2 gene were grown on 24-well cell culture plates ( $8 \times 10^5$  cells per well) with 1.9 cm<sup>2</sup> culture area per well (Cellstar<sup>®</sup>, Greiner Bio-One, Frickenhausen, Germany) in 1 ml DMEM culture medium until confluence. Before testing, the DMEM culture medium was removed and 450 µl of fresh DMEM with 50 µM torasemide or 50 µM furosemide was added. Every value was determined in duplicate. After a preincubation step of 60 min at 37°C, the reaction was started by the addition of 50 µl of DMEM containing the substrate 11-deoxycorticosterone (containing 0,15 µCi of [1,2-<sup>3</sup>H] 11-deoxycorticosterone, dissolved in ethanol, final concentration 100 nM) and cells were incubated for further 45 min. Controls

were treated in the same way without inhibitor. The maximum DMSO concentration in each well was 1 %. Enzyme reaction was stopped by extracting the supernatant with ethyl acetate. Samples were centrifuged (10000 x g, 10 min), and the solvent was pipetted into fresh cups. The solvent was evaporated and the steroids were redissolved in 40  $\mu$ l of methanol and analyzed by HPLC (the conversion of [3H]DOC to [3H]corticosterone and [3H]aldosterone) .

## **2.8. Nuclear fraction extraction**

Cells were harvested and resuspended in 600 $\mu$ l cytosolic lysis buffer (in mmol/L: Hepes 10 [pH 7.9], KCl 10, EDTA 0.1, EGTA 0.1, DTT 1, PMSF 0.5) [30]. After 15 min of swelling, 25  $\mu$ l of 10% NP40 was added. The nuclear pellet was resuspended in 200  $\mu$ l nuclear lysis buffer (in mmol/L: Hepes 20 [pH 7.9], NaCl 0.4, EDTA 1, EGTA 1, DTT 1, PMSF 1). 75  $\mu$ g of protein from nuclear extracts was separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (0.2  $\mu$ m pore size, Schleicher and Schuell, Dassel, Germany).

## **2.9. Quantification of aldosterone concentrations**

The left atrial (LA) tissues samples from FVBN-mice, RacET-mice, RacET-mice (fed with torasemide or furosemide) were weighed and homogenized in 500  $\mu$ l PBS. The protein content was determined using Bradford reagent followed by extraction of aldosterone from homogenates with 1 ml ethyl acetate. The homogenate was vortexed for 15 min and centrifuged for 5 min at 12,500 rpm. The organic layer was separated and evaporated to dryness. Subsequently the samples were redissolved in 50  $\mu$ l PBS. Aldosterone levels in LA were quantified using Homogenous Time Resolved Fluorescence Resonance Energy Transfer (HTR-FRET) with the help of an aldosterone HTRF assay kit (Cisbio Bioassays, France), according to the manufacturer's instruction. Serum aldosterone levels were also quantified

using Homogenous Time Resolved Fluorescence Resonance Energy Transfer (HTR-FRET) using the aldosterone HTRF assay kit from Cisbio Bioassays, France.

### **2.10. Nuclear receptor reporter assay**

Human mineralocorticoid receptor activity was assessed using a luciferase reporter gene assay kit (INDIGO Biosciences, State College, PA, USA) according to the manufacturer's instructions. Reporter cells were dispensed into wells of the assay plate and were then immediately dosed with torasemide, furosemide, SL242 and NSC as indicated in the results section. Following overnight incubation, treatment media were discarded and luciferase detection reagent was added. The intensity of light emission from each sample well was quantified using a plate-reading luminometer.

### **2.11. Histological Analyses**

10 µm cryo-sections were stained with 0.1% Sirius Red F3BA (Polysciences). Lucia Measurement Version 4.6 software was used for quantification of interstitial fibrosis.

### **2.12. Measurement of Hydroxyprolin**

Hydroxyproline concentrations in the supernatants of cultured cardiac fibroblasts were determined by enzyme-linked immunosorbent assay using a hydroxyproline assay kit (Quickzyme, The Netherlands).

### **2.13. Statistical Analysis**

Band intensities were analysed by densitometry. Mean, standard error of the mean (SEM) and statistical analyses were calculated using Sigma Plot 12.3 software version 2.0. For multiple comparisons, ANOVA followed by Newman-Keuls post-hoc analysis for multiple

comparisons was applied. The Fisher exact test was used to compare the prevalence of AF (Graph Pad Priss 5 software). Differences were considered significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Torasemide Reduces CTGF-, LOX- and miR-21 Expression in Cardiac Fibroblasts

Western Blot analysis of neonatal cardiac fibroblasts treated with torasemide or furosemide (50  $\mu\text{M}$ , 24 hours) compared to vehicle showed that torasemide but not furosemide reduced the expression of CTGF ( $65 \pm 6\%$ ,  $p < 0.05$ ) and LOX ( $57 \pm 8\%$ ,  $p < 0.05$ ) whereas the expression of Rac1 GTPase remained unaffected (**Figure 1 A-D**). The regulation of CTGF and LOX did not differ between atrial and ventricular fibroblasts.

Treatment with torasemide reduced miR-21 expression ( $44 \pm 23\%$ ,  $p < 0.05$ ) compared to vehicle-treated cells which was associated with a non-significant trend towards reduced collagen content in torasemide treated neonatal cardiac fibroblasts ( $75 \pm 11\%$ ,  $p = \text{ns}$ ). In contrast, furosemide treatment increased collagen to  $151 \pm 12\%$ ,  $p < 0.05$  (**Figure 1 E+F**).

#### 3.2. Torasemide Reduces CYP11B2 Activity

In order to characterize the underlying cellular mechanism, we investigated the effect of torasemide and furosemide treatment (50  $\mu\text{M}$ , 24 hours) on the mineralocorticoid receptor (MR) and the aldosterone synthase (CY11B2) expression in cardiac fibroblasts. Both loop diuretics did not alter the MR expression. Similarly, luciferase reporter gene assays showed no change of the activity of the MR. Furthermore, the mRNA expression of CYP11B2 was not changed by either one of the two loop diuretics (**Figure 2 A-E**).

CYP450 inhibitors typically contain nitrogen-containing aliphatic or aromatic functions. Therefore, we hypothesized that torasemide may have an inhibitory effect on aldosterone

synthase and that furosemide would not inhibit CYP11B2, as it contains not heterocyclic nitrogen. To test the inhibition of torasemide on CYP11B2 activity, lung fibroblasts (V79MZ cells) that exhibit no endogenous CYP450 activity underwent stable transfection with human aldosterone synthase (CYP11B2) and were treated with torasemide. These experiments showed that torasemide 50  $\mu$ M potently reduces CYP11B2 activity by  $75\pm 1.8\%$  ( $p < 0.05$ ) compared to untreated control. In contrast, furosemide 50  $\mu$ M as well as higher concentrations had no effect on CYP11B2 activity (**Figure 2 F**). Since 50 $\mu$ M torasemide exerted the greatest reduction of CYP11B2 activity, this concentration was used for further *in vitro* experiments.

### **3.3. Selective Inhibition of CYP11B2 reduces Expression of CTGF, LOX, miR-21 and Collagen Content in Cardiac Fibroblasts**

Recently, we developed aldosterone synthase inhibitors with improved activity and selectivity against other steroidogenic enzymes such as CYP11B1, CYP17 and CYP19 as well as hepatic cytochrome P450 enzymes (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) [31-33]. Therefore we studied the effect of specific inhibition of CYP11B2 by using the novel selective inhibitor SL 242 (for detailed information see compound 7 from [34]) in neonatal cardiac fibroblasts. The  $IC_{50}$  of SL242 on the human enzyme *in vitro* was 0.2 nM. The selectivity toward the cortisol forming enzyme CYP11B1 was high:  $IC_{50} = 33$  nM, selectivity factor = 187. The  $IC_{50}$  values toward other steroidogenic or hepatic CYP enzymes were in the micromolar range. Furthermore, SL242 showed a moderate inhibitory effect on rat CYP11B2 ( $IC_{50} = 1077$  nM) *in vitro* and reasonable pharmacokinetic properties<sup>43</sup>. It diminished the aldosterone formation in rat adrenals and significantly reduced plasma aldosterone levels *in vivo*<sup>31</sup>.

To test the effect of selective CYP11B2 inhibition, cardiac fibroblasts were stimulated with angiotensin II (Ang, 1 $\mu$ M; 3 hours) and pre-incubated with or without the CYP11B2 specific

inhibitor SL 242 [34]. The CYP11B2 inhibitor (1 $\mu$ M; 24 hours) reduced the angiotensin induced expression of CTGF (Ang 170 $\pm$ 15% vs. Ang+SL242 118 $\pm$ 9%,  $p$ <0.05), LOX (Ang 218 $\pm$ 47% vs. Ang+SL242 98 $\pm$ 2%,  $p$ <0.05) and the fibrosis regulator microRNA-21 (Ang 217 $\pm$ 40 vs. Ang+SL242 121 $\pm$ 17%,  $p$ <0.05) as well as the collagen content (Ang 312 $\pm$ 52% vs. Ang+SL242 118 $\pm$ 23%,  $p$ <0.05), whereas MCR activity quantified by MR nuclear translocation and luciferase reporter gene assay was unaffected (**Figure 3 A-G**).

### **3.4. Torasemide Reduces CTGF Expression without Affecting Rac1 Activity in Cardiomyocytes**

To further study the underlying mechanisms, neonatal cardiomyocytes were treated with torasemide and furosemide (50  $\mu$ M, 24 hours) and expression of CTGF and Rac1 as well as Rac1 activity was investigated. Torasemide but not furosemide reduced CTGF (42 $\pm$ 10%,  $p$ <0.05) expression (**Figure 4 A**), whereas Rac1 expression and activity were unaffected (**Figure 4 B+C**). Torasemide treatment did not alter CYP11B2 or MR expression but furosemide increased the expression of aldosterone synthase (206 $\pm$ 29%,  $p$ <0.05) (**Figure 4 E+F**). The regulation of CTGF and LOX did not differ between atrial and ventricular fibroblasts and was confirmed in HL-1 cells.

### **3.5. Torasemide Reduces Myocardial Aldosterone Concentration, Atrial Fibrosis and Atrial Fibrillation *in vivo***

To characterize the interaction of aldosterone synthase inhibition and CTGF, LOX, Rac1, miR-21 and atrial fibrosis during AF *in vivo*, transgenic mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the  $\alpha$ MHC promoter (RacET) were studied because these mice develop spontaneous AF at high age [19-21, 25]. RacET mice were treated long-term with torasemide (10mg/kg/day) or furosemide (40mg/kg/day) for 8 months. Furosemide was used at a higher concentration because the diuretic potency of

torasemide is known to be 4 fold higher than furosemide [2, 3, 35]. Pilot experiments had shown that these doses yield similar urine output in mice.

The characteristics of the mice are depicted in **table 1**. Both diuretics exerted similar effects on urine output, but renal sodium, potassium and calcium output were increased in furosemide treated mice compared to untreated or torasemide treated RacET, whereas serum electrolyte levels were unaffected. Blood pressure, left ventricular function and left atrial size were comparable between the treated animals.

WT and untreated RacET exhibited similar serum aldosterone levels, whereas torasemid treatment showed a non-significant trend towards reduced and furosemide a non-significant trend towards increased serum aldosterone levels (**Figure 5 A**). Aldosterone concentrations were increased in atria of RacET mice compared to WT. This effect was prevented by torasemide but not by furosemide treatment (**Figure 5 B**). MR and CYP11B2 expression were unaffected by both loop diuretics (**Figure 5 C-E**).

The RacET mice were characterized by a 3fold increase of atrial fibrosis ( $p < 0.05$ ), increased protein expression of CTGF ( $257 \pm 77\%$ ,  $p < 0.05$ ), LOX ( $195 \pm 24\%$ ,  $p < 0.05$ ) and miR-21 ( $252 \pm 43\%$ ,  $p < 0.05$ ) compared to wildtype mice (WT) (**Figure 6 A-F**). Treatment with torasemide for 8 months - but not with furosemide - prevented atrial fibrosis in RacET. Similarly, torasemide prevented the up-regulation of CTGF ( $62 \pm 18\%$ ,  $p < 0.05$ ), LOX ( $124 \pm 23\%$ ,  $p < 0.05$ ) and miR-21 ( $68 \pm 7\%$ ,  $p < 0.05$ ) compared to vehicle or to furosemide (**Figure 6 A-F**). Importantly, torasemide reduced the prevalence of atrial fibrillation (33% RacET+Tora vs. 80% RacET,  $p < 0.05$ ) (**Figure 6 E**).

#### 4. Discussion

The data identify the aldosterone synthase (CYP11B2) as a key regulator of fibrotic remodeling during atrial fibrillation. Aldosterone synthase regulates miR-21 via the pro-fibrotic mediators connective tissue growth factor and lysyl oxidase down-stream of Rac1 GTPase. The loop diuretic torasemide reduces aldosterone synthase activity and prevents atrial fibrosis and atrial fibrillation in mice.

Aldosterone synthase (CYP11B2) catalyses the three terminal steps in the biosynthesis of the mineralocorticoid aldosterone [36]. It is the only enzyme capable of synthesizing aldosterone in humans. CYP11B2 is a mitochondrial cytochrome P450 enzyme which is located in the inner mitochondrial membrane, mainly in the *Zona glomerulosa* of the adrenal cortex and to a much lesser extent in the myocardium [37-40]. Here, our data confirm the expression and functional role of CYP11B2 in cardiac myocytes and in cardiac fibroblasts [41]. By binding to epithelial mineralocorticoid receptors (MR), aldosterone plays an important role regulating sodium and water retention as well as potassium secretion. In addition, the MR is a direct regulator of left ventricular myocardial remodeling [42]. Aldosterone release is triggered by angiotensin-II, the extracellular potassium concentration and adrenocorticotrophic hormones [43, 44]. Aldosterone directly contributes to the pathogenesis of fibrosis and AF [10-15, 26, 38]. The small GTPase-Rac1, which contributes to the generation of reactive oxygen species (ROS) by coupling to NOX1 [45], the connective tissue growth factor, the lysyl oxidase and miR-21 contribute to the aldosterone-induced signal transduction during the pathogenesis of atrial remodeling [19-21, 25] + [26]. Here, the experiments in primary cardiac fibroblasts and cardiomyocytes show that torasemide but not furosemide reduces the expression of CTGF, LOX and miR-21. The effects are down-stream of Rac1 expression and activity. Importantly, the study identifies inhibition of the aldosterone synthase by torasemide as important underlying mechanism.



Similar to other members of the cytochrome P450 family, the aldosterone synthase contains a heme as prosthetic group. Effective CYP450 inhibitors simultaneously bind to lipophilic regions on the active site and to the 6th position of the pentacoordinated heme iron. They inactivate redox function of the CYP450 and competitively inhibit binding of the substrate dioxygen. The structures of these inhibitors usually incorporate nitrogen-containing aliphatic or aromatic functions that mediate the coordination to the heme iron [32-34]. Our experiments in lung fibroblasts deficient of endogenous aldosterone synthase transfected with human CYP11B2 show that treatment with torasemide but not furosemide markedly inhibits CYP11B2 activity by 75%. A potential mechanism may relate to competitive inhibition of CYP11B2 by binding of torasemide to the heme binding site of CYP11B2 through its heterocyclic nitrogen. This hypothesis is visualized in **Supplement Figure1**. The inhibition of aldosterone synthase contributes to the signal transduction of structural remodelling in left atrial myocardium leading to interstitial fibrosis.

The experimental data are supported by clinical evidence that suggest differential cardiac effects of loop diuretics. The TORIC (Torasemide In Congestive Heart Failure) study, an open-label study of 1,337 patients with New York Heart Association class II to III HF, was the largest study comparing furosemide with newer loop diuretics. TORIC showed that a greater proportion of patients receiving torsemide improved their functional class (45.8% vs. 37.2%,  $p < 0.00017$ ) and that fewer patients receiving torsemide died (2.2% vs. 4.5%,  $p < 0.05$ ) [5]. Additionally, a recent meta-analysis suggests improved functional status and mortality with torsemide compared with furosemide [46].

A crucial point in the development of any CYP inhibitor is selectivity. This is especially true of CYP11B2 since the inhibitors must not affect 11 $\beta$  hydroxylase (CYP11B1), which is the

key enzyme of glucocorticoid biosynthesis. Selectivity towards aldosterone synthase is not easy to reach, because CYP11B1 and CYP11B2 exhibit a sequence homology of 93%. SL242 is a non-steroidal compound in order to minimize the risk of side effects caused by interaction with steroid receptors or other steroidogenic enzymes and is the only compound, which is able to inhibit human CYP11B2 to 94% [0.5  $\mu$ M] and rat CYP11B2 to 65% [2  $\mu$ M][34]. Thus, SL242 is a suitable tool for studies in disease-oriented models as described in this paper. Indeed, the data show that CYP11B2 inhibition by SL 242 completely prevented angiotensin II induced CTGF, LOX and miR-212 expression as well as angiotensin II induced fibrosis, without affecting MR activity.

To further assess the relation of myocardial Rac1, aldosterone synthase and atrial fibrosis and atrial fibrillation *in vivo*, transgenic mice over-expressing Rac1 under the control of the MHC promoter which develop atrial fibrillation at older age [19-21, 29] were treated for 8 months with torasemide or furosemide [2, 3, 35]. Both diuretics exerted similar effect on urine output as well as on blood pressure, left ventricular function and left atrial size (**Table 1**; [2, 3]). The main novel finding of the experiment is that only torasemide-treated animals exhibited a reduction in atrial fibrosis. Importantly, this finding correlated with a marked reduction of the phenotype of spontaneous atrial fibrillation.

The RacET mice are characterized by up-regulation of CTGF, LOX, miR-21 and fibrosis in the left atrium. Furthermore, RacET mice exhibit a marked increase of myocardial aldosterone tissue concentrations, which was completely prevented by torasemide- but not by furosemide treatment. Compared to their wildtypes, RacET mice show similar aldosterone serum- but increased myocardial aldosterone concentrations which were reduced by torasemide. This finding may be explained by the reported ability of torasemide to reduce trans-cardiac extraction of circulating aldosterone [47] and/or by the direct inhibition of

aldosterone synthase activity. The data are in agreement with previous studies reporting that torasemide is not an MR antagonist [41] but reduces the expression of aldosterone synthase and fibrosis in the left ventricles of rats with dilated cardiomyopathy [35, 48] as well as the incidence of atrial fibrillation in patients with dilated cardiomyopathy [49]. In agreement with our cell culture data, CYP11B2 inhibition by torasemide in RacET correlated with a potent reduction of CTGF, LOX, miR-21 and atrial fibrosis *in vivo* without affecting MR or CYP11B2 expression supporting the proposed mechanism of action. Taken together, torasemide exerts beneficial direct structural and functional effects on atrial remodeling in this mouse model.

The study has some limitations. First ventricular rat neonatal cells have limitations because atrial fibrillation in vivo is regulated by the interaction of several cell types, mechanical forces and electrical stimulation. In addition, ventricular cells may differ from atrial cells. Therefore, the main findings of the study were confirmed in atrial fibroblasts and in HL-1 cells. Importantly and consistently, the regulation e.g. of LOX and CTGF was identical in neonatal atrial fibroblasts and cardiomyocytes compared to the ventricular fibroblasts as well as in HL-1 cells (Supplement Figure 2). In our study, cultured cells are applied to obtain mechanistic insight by enhancing and inhibiting specific steps in the signal cascade. However, in order to gain information about the *in vivo* situation, the mouse model was studied. For future experiments, inhibition of CYP11B2 *in vivo* by SL 242 would be of interest. However, the specific CYP11B2 inhibitors show high species selectivity and SL242 may be specific for rat and humans but not mice. In addition, the synthesis of the amount of SL242 needed for the long term *in vivo* studies represents a challenge. SL242 was therefore used for the mechanistic experiments to explain the observations obtained with torasemide, which has the advantage to be widely available including clinical use. Clearly, our data support the concept of studying novel inhibitors of aldosterone synthase in humans [51].

## 5. Conclusion

In summary, torasemide but not furosemide reduces aldosterone synthase mediated pro-fibrotic signalling, which induces miR-21 expression, CTGF and LOX. These mechanistic studies set the stage to evaluate the effects of aldosterone synthase inhibition on atrial remodeling in humans. The improved understanding of the signalling during atrial fibrosis may help to identify novel therapeutic targets such as the aldosterone synthase.

## 6. Perspectives

The prognostic effect of loop diuretics may differ between torasemide and furosemide. Only torasemide reduces aldosterone synthase mediated pro-fibrotic signalling in mice and prevents atrial fibrillation. These mechanistic studies set the stage to evaluate the effects of torasemide and of aldosterone synthase inhibition on atrial remodeling in humans.

## 7. Acknowledgments

We thank Simone Jäger and Ellen Becker for their excellent technical assistance.

### References

- [1] McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J*. 2012;33:1787-847.
- [2] Broekhuysen J, Deger F, Douchamps J, Ducarne H, Herchuelz A. Torasemide, a new potent diuretic. Double-blind comparison with furosemide. *Eur J Clin Pharmacol*. 1986;31 Suppl:29-34.
- [3] Murray MD, Deer MM, Ferguson JA, Dexter PR, Bennett SJ, Perkins SM, et al. Open-label randomized trial of torsemide compared with furosemide therapy for patients with heart failure. *Am J Med*. 2001;111:513-20.
- [4] Lopez B, Querejeta R, Gonzalez A, Sanchez E, Larman M, Diez J. Effects of loop diuretics on myocardial fibrosis and collagen type I turnover in chronic heart failure. *J Am Coll Cardiol*. 2004;43:2028-35.
- [5] Cosin J, Diez J. Torasemide in chronic heart failure: results of the TORIC study. *Eur J Heart Fail*. 2002;4:507-13.

- [6] Allesie M, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. *Cardiovasc Res.* 2002;54:230-46.
- [7] Li D, Fareh S, Leung TK, Nattel S. Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort 1. *Circulation.* 1999;100:87-95.
- [8] Nattel S, Burstein B, Dobrev D. Atrial remodeling and atrial fibrillation: mechanisms and implications. *Circ Arrhythm Electrophysiol.* 2008;1:62-73.
- [9] Nattel S. New ideas about atrial fibrillation 50 years on. *Nature.* 2002;415:219-26.
- [10] Delcayre C, Swynghedauw B. Molecular mechanisms of myocardial remodeling. The role of aldosterone. *J Mol Cell Cardiol.* 2002;34:1577-84.
- [11] Weber KT, Gerling IC, Kiani MF, Guntaka RV, Sun Y, Ahokas RA, et al. Aldosteronism in heart failure: a proinflammatory/fibrogenic cardiac phenotype. Search for biomarkers and potential drug targets. *Curr Drug Targets.* 2003;4:505-16.
- [12] Fraccarollo D, Galuppo P, Hildemann S, Christ M, Ertl G, Bauersachs J. Additive improvement of left ventricular remodeling and neurohormonal activation by aldosterone receptor blockade with eplerenone and ACE inhibition in rats with myocardial infarction. *J Am Coll Cardiol.* 2003;42:1666-73.
- [13] Fraccarollo D, Galuppo P, Schmidt I, Ertl G, Bauersachs J. Additive amelioration of left ventricular remodeling and molecular alterations by combined aldosterone and angiotensin receptor blockade after myocardial infarction. *Cardiovasc Res.* 2005;67:97-105.
- [14] Reil JC, Hohl M, Selejan S, Lipp P, Drautz F, Kazakow A, et al. Aldosterone promotes atrial fibrillation. *Eur Heart J.* 2012;33:2098-108.
- [15] Ackland ML, Newgreen DF, Fridman M, Waltham MC, Arvanitis A, Minichiello J, et al. Epidermal growth factor-induced epithelio-mesenchymal transition in human breast carcinoma cells. *Lab Invest.* 2003;83:435-48.
- [16] Yamato M, Sasaki T, Honda K, Fukuda M, Akutagawa O, Okamoto M, et al. Effects of torasemide on left ventricular function and neurohumoral factors in patients with chronic heart failure. *Circ J.* 2003;67:384-90.
- [17] Goodfriend TL, Ball DL, Oelkers W, Bahr V. Torsemide inhibits aldosterone secretion in vitro. *Life Sci.* 1998;63:PL45-50.
- [18] Uchida T, Yamanaga K, Nishikawa M, Ohtaki Y, Kido H, Watanabe M. Anti-aldosteronergic effect of torasemide. *Eur J Pharmacol.* 1991;205:145-50.
- [19] Adam O, Frost G, Custodis F, Sussman MA, Schäfers HJ, Böhm M, et al. Role of Rac1 GTPase activation in atrial fibrillation. *J Am Coll Cardiol.* 2007;50:359-67.
- [20] Adam O, Lavall D, Theobald K, Hohl M, Grube M, Ameling S, et al. Rac1-induced connective tissue growth factor regulates connexin 43 and N-cadherin expression in atrial fibrillation. *J Am Coll Cardiol.* 2010;55:469-80.
- [21] Adam O, Theobald K, Lavall D, Grube M, Kroemer HK, Ameling S, et al. Increased lysyl oxidase expression and collagen cross-linking during atrial fibrillation. *J Mol Cell Cardiol.* 2011;50:678-85.
- [22] Lopez B, Gonzalez A, Hermida N, Valencia F, de Teresa E, Diez J. Role of lysyl oxidase in myocardial fibrosis. From basic science to clinical aspects. *Am J Physiol Heart Circ Physiol.* 2010;299:H1-9.
- [23] Lopez B, Querejeta R, Gonzalez A, Beaumont J, Larman M, Diez J. Impact of treatment on myocardial lysyl oxidase expression and collagen cross-linking in patients with heart failure. *Hypertension.* 2009;53:236-42.
- [24] Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature.* 2008;456:980-4.
- [25] Adam O, Löhlfelm B, Thum T, Gupta SK, Puhl SL, Schäfers HJ, et al. Role of miR-21 in the pathogenesis of atrial fibrosis. *Basic Res Cardiol.* 2012;107:278.

- [26] Lavall D, Selzer C, Schuster P, Lenski M, Adam O, Schäfers HJ, et al. The mineralocorticoid receptor promotes fibrotic remodeling in atrial fibrillation. *J Biol Chem*. 2014;289:6656-68.
- [27] Custodis F, Eberl M, Kilter H, Böhm M, Laufs U. Association of RhoGDIalpha with Rac1 GTPase mediates free radical production during myocardial hypertrophy. *Cardiovasc Res*. 2006;71:342-51.
- [28] Sussman MA, Welch S, Walker A, Klevitsky R, Hewett TE, Price RL, et al. Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. *J Clin Invest*. 2000;105:875-86.
- [29] Reil JC, Hohl M, Oberhofer M, Kazakov A, Kaestner L, Mueller P, et al. Cardiac Rac1 overexpression in mice creates a substrate for atrial arrhythmias characterized by structural remodelling. *Cardiovasc Res*. 2010;87:485-93.
- [30] Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res*. 1989;17:6419.
- [31] Heim R, Lucas S, Grombein CM, Ries C, Schewe KE, Negri M, et al. Overcoming undesirable CYP1A2 inhibition of pyridynaphthalene-type aldosterone synthase inhibitors: influence of heteroaryl derivatization on potency and selectivity. *J Med Chem*. 2008;51:5064-74.
- [32] Lucas S, Heim R, Negri M, Antes I, Ries C, Schewe KE, et al. Novel aldosterone synthase inhibitors with extended carbocyclic skeleton by a combined ligand-based and structure-based drug design approach. *J Med Chem*. 2008;51:6138-49.
- [33] Lucas S, Heim R, Ries C, Schewe KE, Birk B, Hartmann RW. In vivo active aldosterone synthase inhibitors with improved selectivity: lead optimization providing a series of pyridine substituted 3,4-dihydro-1H-quinolin-2-one derivatives. *J Med Chem*. 2008;51:8077-87.
- [34] Ries C, Lucas S, Heim R, Birk B, Hartmann RW. Selective aldosterone synthase inhibitors reduce aldosterone formation in vitro and in vivo. *J Steroid Biochem Mol Biol*. 2009;116:121-6.
- [35] Veeraveedu PT, Watanabe K, Ma M, Thandavarayan RA, Palaniyandi SS, Yamaguchi K, et al. Comparative effects of torasemide and furosemide in rats with heart failure. *Biochem Pharmacol*. 2008;75:649-59.
- [36] Kawamoto T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S, et al. Role of steroid 11 beta-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *Proc Natl Acad Sci U S A*. 1992;89:1458-62.
- [37] Silvestre JS, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic JM, et al. Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. *J Biol Chem*. 1998;273:4883-91.
- [38] Delcayre C, Silvestre JS. Aldosterone and the heart: towards a physiological function? *Cardiovasc Res*. 1999;43:7-12.
- [39] Young MJ, Clyne CD, Cole TJ, Funder JW. Cardiac steroidogenesis in the normal and failing heart. *J Clin Endocrinol Metab*. 2001;86:5121-6.
- [40] Satoh M, Nakamura M, Saitoh H, Satoh H, Akatsu T, Iwasaka J, et al. Aldosterone synthase (CYP11B2) expression and myocardial fibrosis in the failing human heart. *Clin Sci (Lond)*. 2002;102:381-6.
- [41] Gravez B, Tarjus A, Jimenez-Canino R, El Moghrabi S, Messaoudi S, Alvarez de la Rosa D, et al. The diuretic torasemide does not prevent aldosterone-mediated mineralocorticoid receptor activation in cardiomyocytes. *PLoS One*. 2013;8:e73737.
- [42] Fraccarollo D, Berger S, Galuppo P, Kneitz S, Hein L, Schutz G, et al. Deletion of cardiomyocyte mineralocorticoid receptor ameliorates adverse remodeling after myocardial infarction. *Circulation*. 2011;123:400-8.

- [43] Davila DF, Nunez TJ, Odreman R, de Davila CA. Mechanisms of neurohormonal activation in chronic congestive heart failure: pathophysiology and therapeutic implications. *Int J Cardiol.* 2005;101:343-6.
- [44] Briet M, Schiffrin EL. Aldosterone: effects on the kidney and cardiovascular system. *Nat Rev Nephrol.* 2010;6:261-73.
- [45] Cheng G, Diebold BA, Hughes Y, Lambeth JD. Nox1-dependent reactive oxygen generation is regulated by Rac1. *J Biol Chem.* 2006;281:17718-26.
- [46] Bikdeli B, Strait KM, Dharmarajan K, Partovian C, Coca SG, Kim N, et al. Dominance of furosemide for loop diuretic therapy in heart failure: time to revisit the alternatives? *J Am Coll Cardiol.* 2013;61:1549-50.
- [47] Tsutamoto T, Sakai H, Wada A, Ishikawa C, Ohno K, Fujii M, et al. Torasemide inhibits transcardiac extraction of aldosterone in patients with congestive heart failure. *J Am Coll Cardiol.* 2004;44:2252-3.
- [48] Veeraveedu PT, Watanabe K, Ma M, Palaniyandi SS, Yamaguchi K, Suzuki K, et al. Torasemide, a long-acting loop diuretic, reduces the progression of myocarditis to dilated cardiomyopathy. *Eur J Pharmacol.* 2008;581:121-31.
- [49] Han LN, Guo SL, Lin XM, Shi XM, Zang CB, Yang LM, et al. Torasemide reduces dilated cardiomyopathy, complication of arrhythmia, and progression to heart failure. *Genet Mol Res.* 2014;13:7262-74.
- [50] Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A.* 1998;95:2979-84.
- [51] Azizi M, Amar L, Menard J. Aldosterone synthase inhibition in humans. *Nephrol Dial Transplant.* 2013;28:36-43.

## **Figure Legends**

### **Figure 1: Torasemide reduces CTGF, LOX and miR-21 expression**

(A-D) Quantification and representative western blots showing the effect of torasemide (Tora, 50  $\mu$ M, 24 hours) and furosemide (Furo, 50  $\mu$ M, 24 hours) treatment in neonatal rat cardiac fibroblasts on the protein expression of connective tissue growth factor (CTGF), lysyl oxidase (LOX) and Rac1 related to GAPDH, n=5, \*p<0.05 vs. control (C). (E) Quantification of miR-21 expression by TaqMan PCR showing the effect of torasemide (50  $\mu$ M, 24 hours) and furosemide (50  $\mu$ M, 24 hours) treatment in neonatal rat cardiac fibroblasts, n=5, \*p<0.05 vs. control (C). (F) Quantification of collagen content by hydroxyproline assay showing the effect of torasemide (50  $\mu$ M, 24 hours) and furosemide (50  $\mu$ M, 24 hours) treatment in neonatal rat cardiac fibroblasts, n=5, \*p<0.05 vs. control (C).

### **Figure 2: Torasemide reduces CYP11B2 Activity**

(A-C) Quantification and representative RT-PCR showing the effect of torasemide (Tora, 50  $\mu$ M, 24 hours) and furosemide (Furo, 50  $\mu$ M, 24 hours) treatment in neonatal rat cardiac fibroblasts on the mRNA expression of the mineralocorticoid receptor (MR) and aldosterone synthase (CYP11B2) related to GAPDH, n=5, p=ns vs. control (C).

Effects of treatment with aldosterone (Aldo, 10 nM, 3hours), torasemide (Tora, 50  $\mu$ M, 24 hours) and furosemide (Furo, 50  $\mu$ M, 24 hours) on (D) mineralocorticoid receptor (MR) translocation and (E) mineralocorticoid receptor (MR) activity quantified by luciferase transporter assay, n=5, \*p<0.05 vs. control (C); #p<0.05 vs. Aldo. (F) Quantification of CYP11B2 activity showing the effect of torasemide (Tora, 50  $\mu$ M) and furosemide (Furo, 50



$\mu\text{M}$ ) treatment in lung fibroblasts (V97MZ cells) deficient of endogenous aldosterone synthase after transfection with human aldosterone synthase,  $n=5$ ,  $*p<0.05$  vs. control (C).

### **Figure 3: Selective CYP11B2 Inhibition reduces Collagen Content in Neonatal Cardiac Fibroblasts**

**(A-C)** Quantification and representative western blots of connective tissue growth factor (CTGF) and lysyl oxidase (LOX) protein expression related to GAPDH in neonatal cardiac fibroblasts stimulated with angiotensin II (Ang,  $1\mu\text{M}$ ; 3 hours) and pre-incubated with or without the CYP11B2 specific inhibitor SL 242 ( $1\mu\text{M}$ ; 24 hours),  $n=5$ ,  $*p<0.05$  vs. control (C);  $\#p<0.05$  vs. Ang. **(D)** Quantification of miR-21 expression by TaqMan PCR of neonatal cardiac fibroblasts stimulated with angiotensin II (Ang,  $1\mu\text{M}$ ; 3 hours) and pre-incubated with or without the CYP11B2 specific inhibitor SL 242 ( $1\mu\text{M}$ ; 24 hours),  $n=5$ ,  $*p<0.05$  vs. control (C);  $\#p<0.05$  vs. Ang. **(E)** Quantification of nuclear mineralocorticoid receptor (MR) translocation related to polymerase II in neonatal rat cardiac fibroblasts stimulated with angiotensin II (Ang,  $1\mu\text{M}$ ; 3 hours) and pre-incubated with or without the CYP11B2 specific inhibitor SL 242 ( $1\mu\text{M}$ ; 24 hours),  $n=5$ ,  $p=\text{ns}$  vs. control (C). **(F)** Effect of treatment with aldosterone (Aldo, 10 nM, 3hours) and the CYP11B2 specific inhibitor SL 242 ( $1\mu\text{M}$ ; 24 hours) on mineralocorticoid receptor (MR) activity quantified by luciferase transporter assay,  $n=5$ ,  $*p<0.05$  vs. control (C);  $\#p<0.05$  vs. Aldo. **(G)** Quantification of collagen content by hydroxyproline assay showing the effect in neonatal cardiac fibroblasts stimulated with angiotensin II (Ang,  $1\mu\text{M}$ ; 3 hours) and pre-incubated with or without the CYP11B2 specific inhibitor SL 242 ( $1\mu\text{M}$ ; 24 hours),  $n=5$ ,  $*p<0.05$  vs. control (C);  $\#p<0.05$  vs. Ang.

#### **Figure 4: Torasemide does not affect Rac1 Expression or Activity**

(A-D) Quantification and representative western blots showing the effect of torasemide (Tora, 50  $\mu$ M, 24 hours) and furosemide (Furo, 50  $\mu$ M, 24 hours) treatment in neonatal rat cardiomyocytes on the protein expression of connective tissue growth factor (CTGF) and Rac1 related to GAPDH, as well as on Rac1 activity (Rac1-Pak), n=5, \*p<0.05 vs. control (C). (E+F) Quantification and representative RT-PCR showing the effect of torasemide (Tora, 50  $\mu$ M, 24 hours) and furosemide (Furo, 50  $\mu$ M, 24 hours) treatment in neonatal rat cardiomyocytes on the mRNA expression of the mineralocorticoid receptor (MR) and aldosterone synthase (CYP11B2) related to GAPDH, n=5, \*p<0.05 vs. control (C).

#### **Figure 5: Torasemide reduces myocardial Aldosteron Tissue Concentration in vivo**

Transgenic mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the  $\alpha$ -myosin heavy chain (MHC) promoter (RacET) were treated with torasemide 10 mg/kg/d p.o. (RacET+Tora) or 40 mg/kg/d furosemide (RacET+Furo) or regular chow for 8 months and compared to 8 months old wild type controls (WT). (A+B) Quantification of serum and myocardial (atrial) tissue aldosterone levels; n=5 per group, \*p<0.05 vs. WT, #p<0.05 vs. RacET. (C-E) Quantification and representative RT-PCR of mRNA expression of the mineralocorticoid receptor (MR) and aldosterone synthase (CYP11B2) n=5 per group, p=ns.

#### **Figure 6: Inhibition of miR-21 prevents Atrial Fibrosis *in vivo***

Transgenic mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the  $\alpha$ -myosin heavy chain (MHC) promoter (RacET) were treated with torasemide

10 mg/kg/d p.o. (RacET+Tora) or 40 mg/kg/d furosemide (RacET+Furo) or regular chow for 8 months and compared to 8 months old wild type controls (WT). **(A)** Representative Sirius red staining (10x fold magnification) and **(B)** quantification of fibrosis in the left atria, n=3 per group, \*p<0.05 vs WT, #p<0.05 vs RacET. **(C-E)** Quantification and representative western blots of connective tissue growth factor (CTGF) and lysyl oxidase (LOX) protein expression related to GAPDH; n=5 per group, \*p<0.05 vs. WT, #p<0.05 vs. RacET. **(F)** Quantification of miR-21 expression by TaqMan PCR; n=5 per group, \*p<0.05 vs. WT, #p<0.05 vs. RacET. **(G)** Quantification of heart rhythm; n=15 per group, \*p<0.05 vs. WT, #p<0.05 vs. RacET.

#### **Table 1: Mice characteristics**

FS = fractional shortening; IVSd = interventricular septum diastolic; IVSs = interventricular septumsystolic; LA = left atrium; LVEDd = left ventricular end-diastolic diameter; LVESd =left ventricular end-systolic diameter; LVPWd = left posterior wall diastolic; LVPWs = left posterior wall systolic.

Figure 1

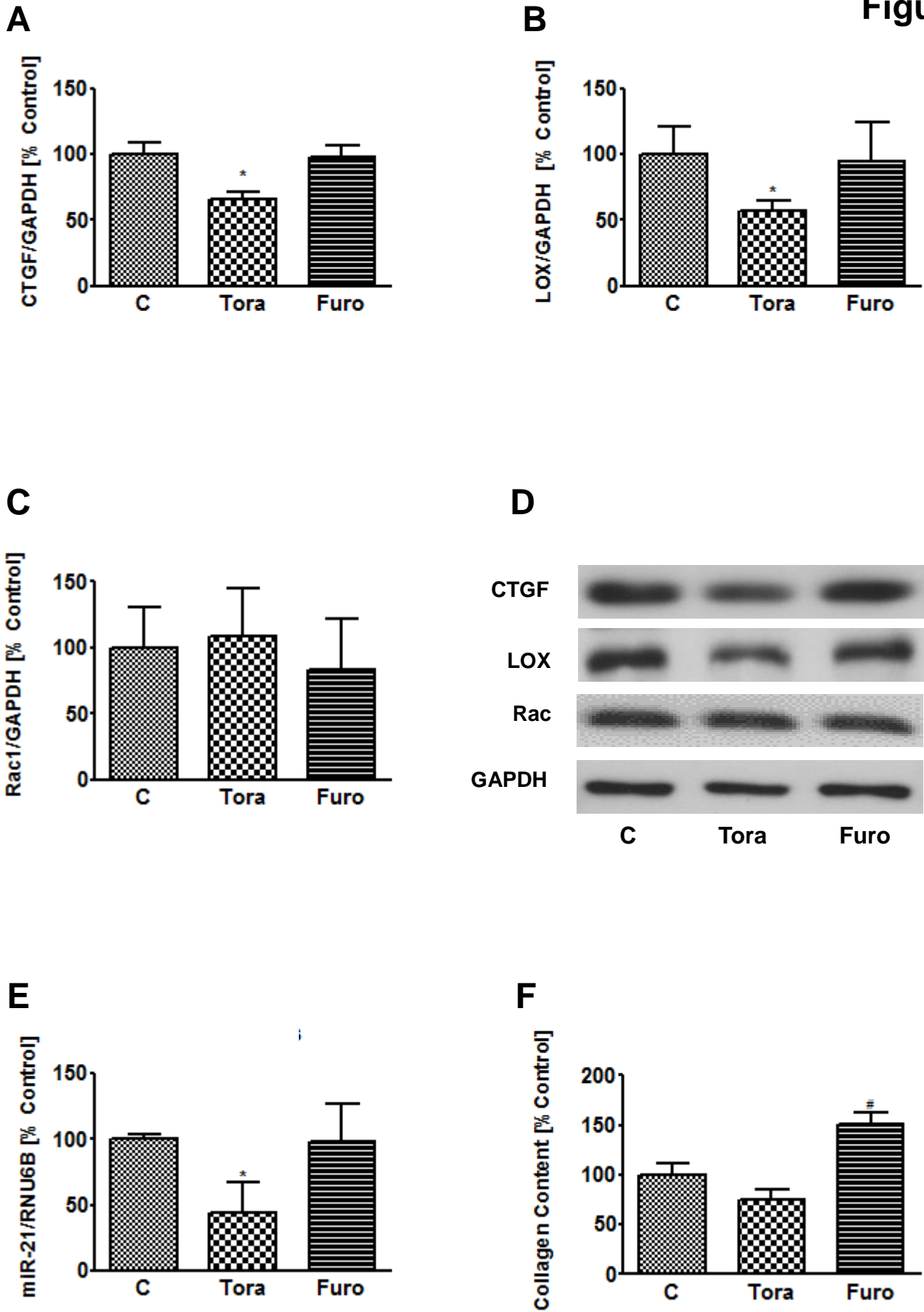
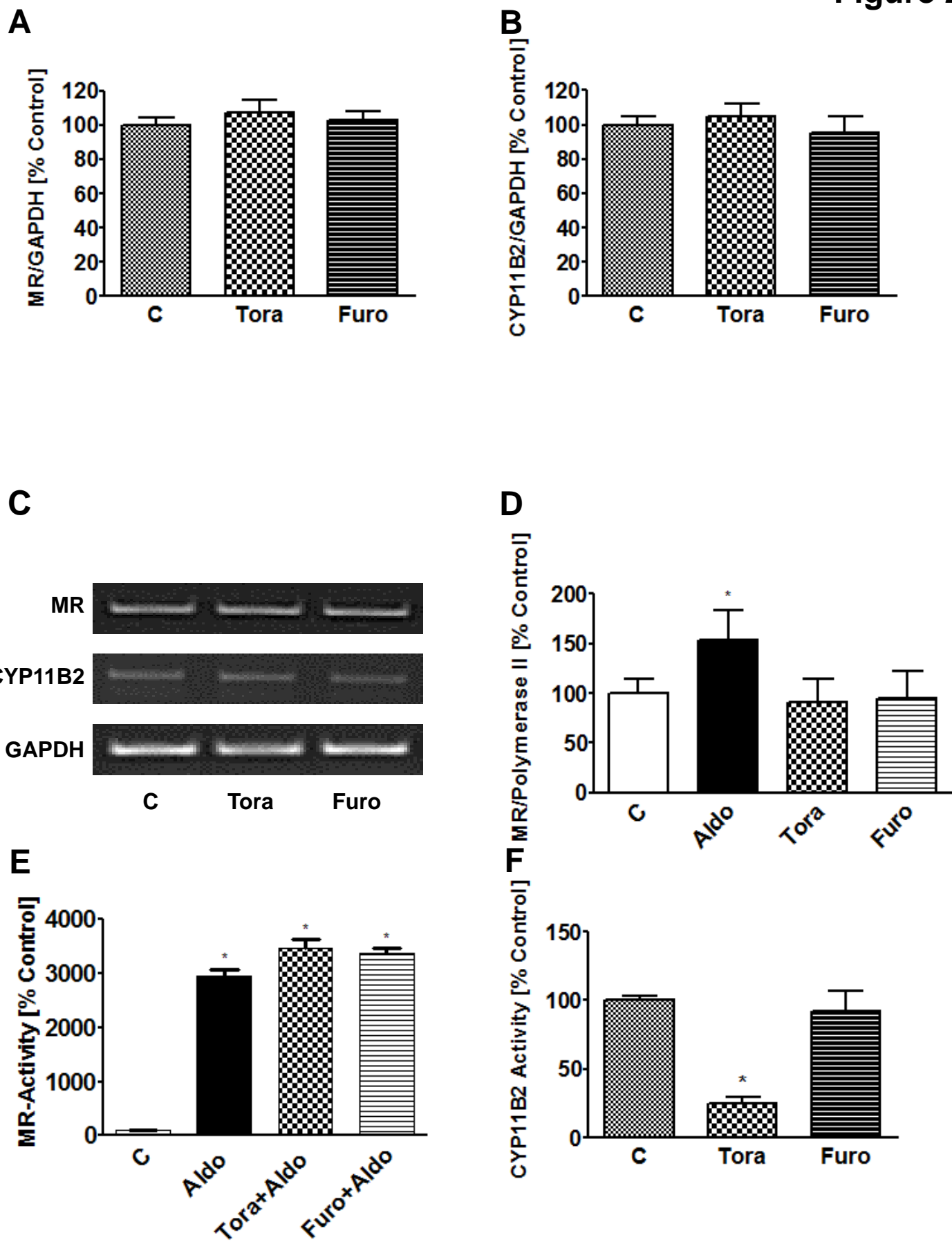


Figure 2



**Figure 3**

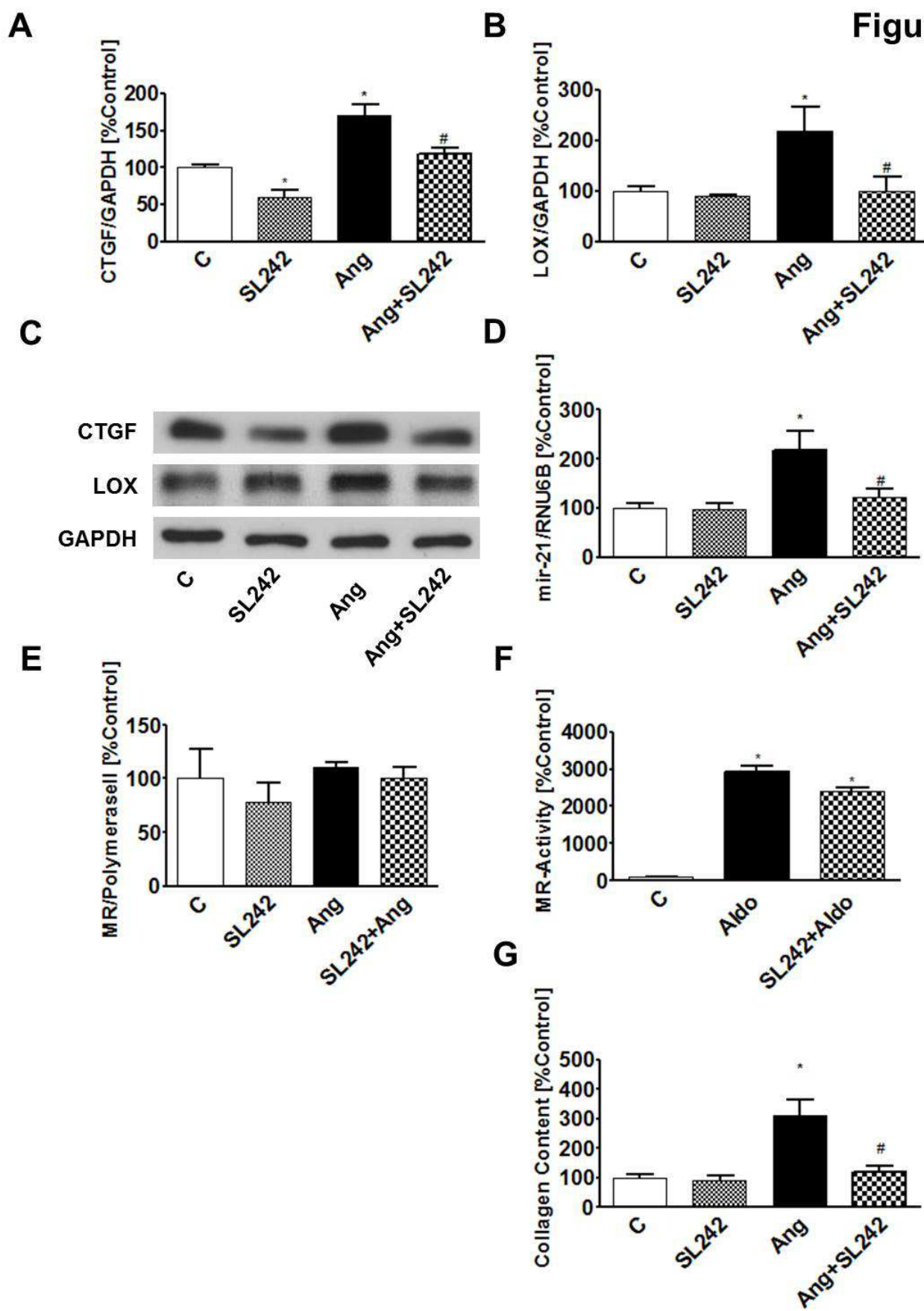


Figure 4

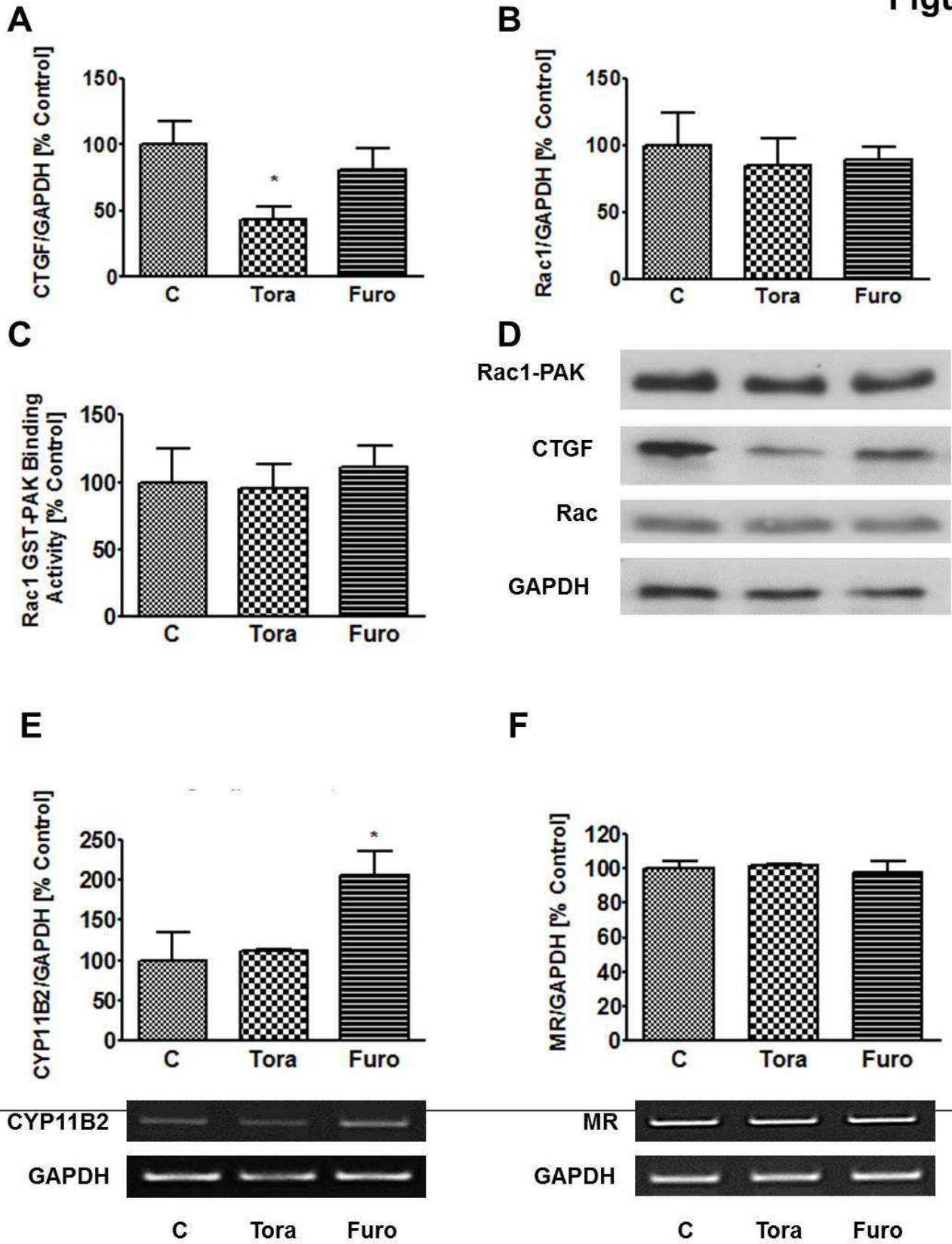
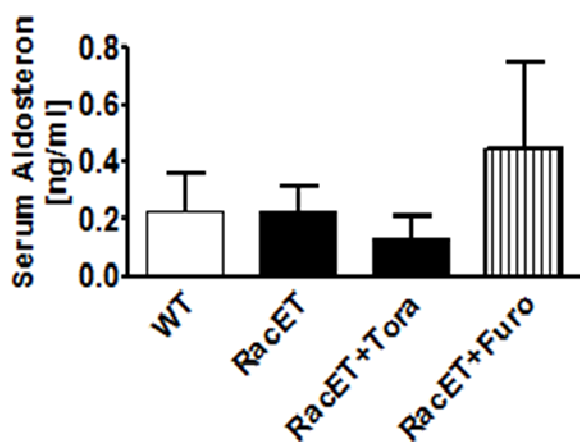
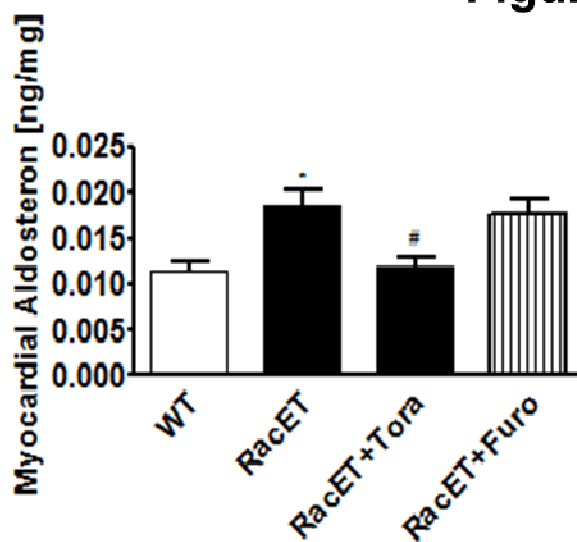


Figure 5

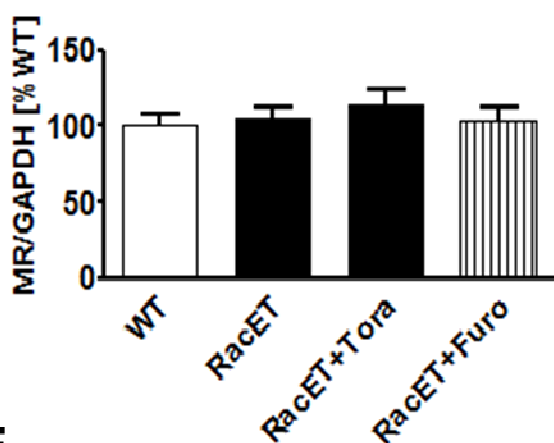
A



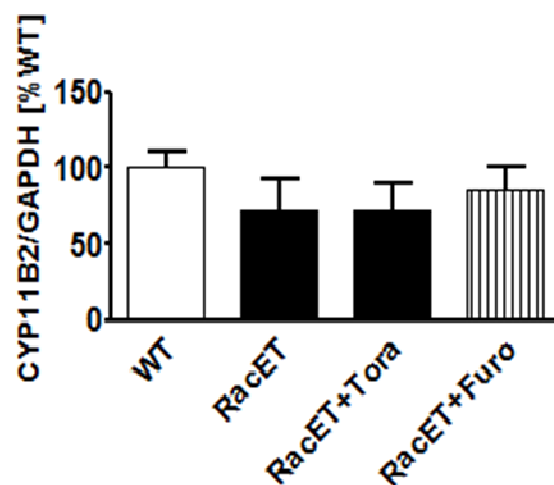
B



C



D



E

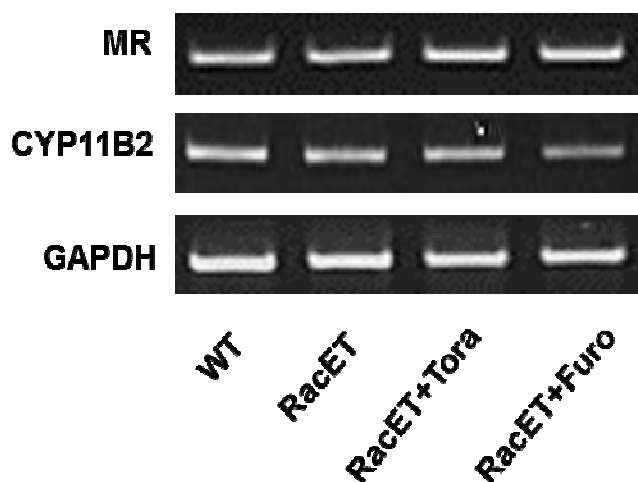
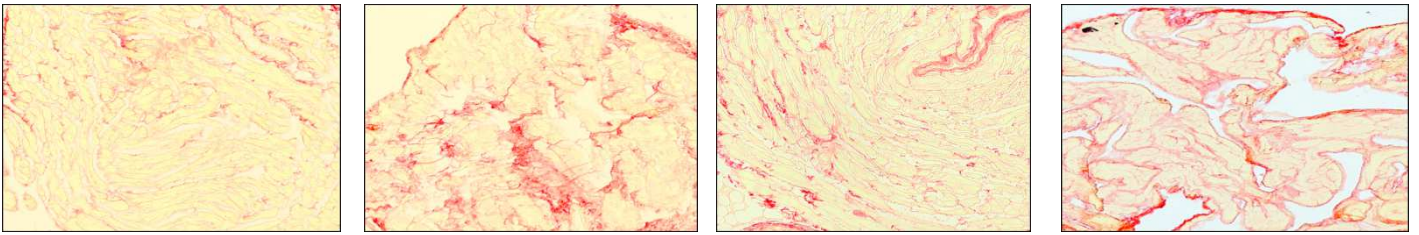


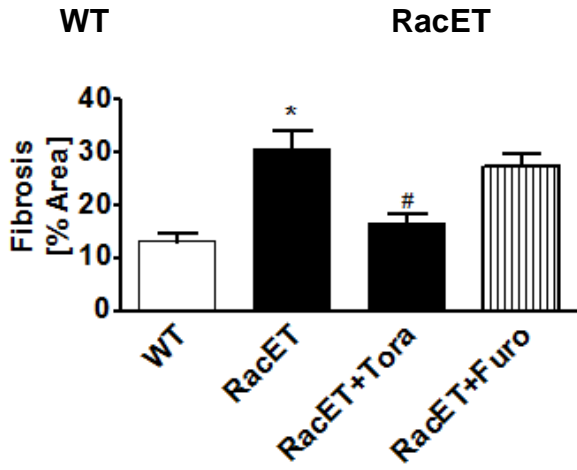


Figure 6

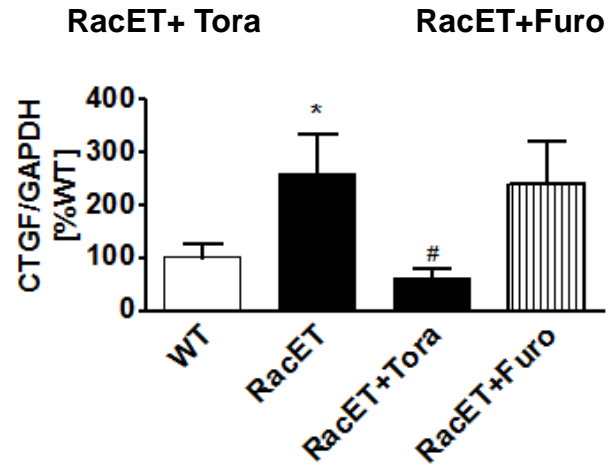
A



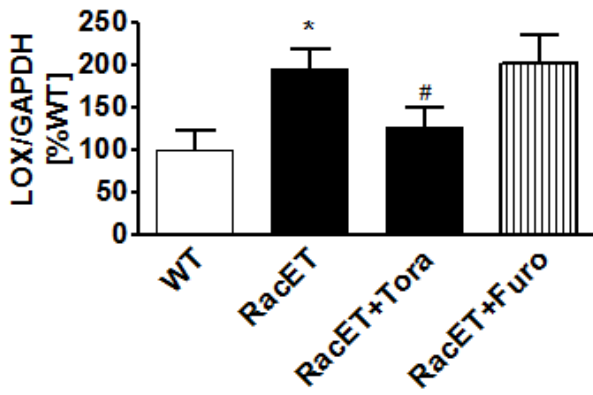
B



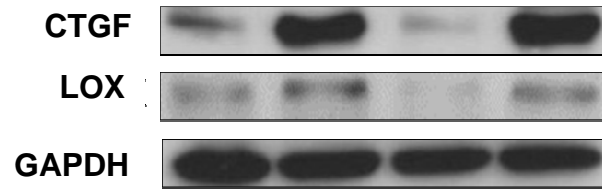
C



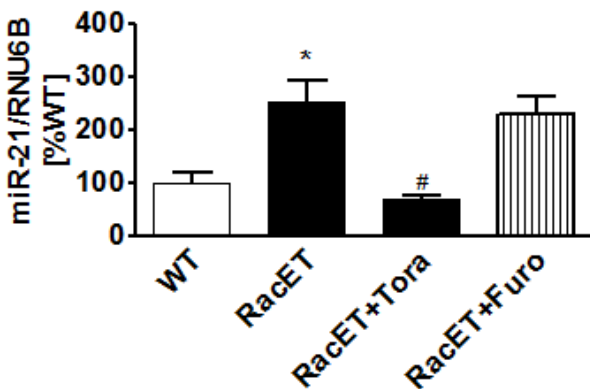
D



E



F



G

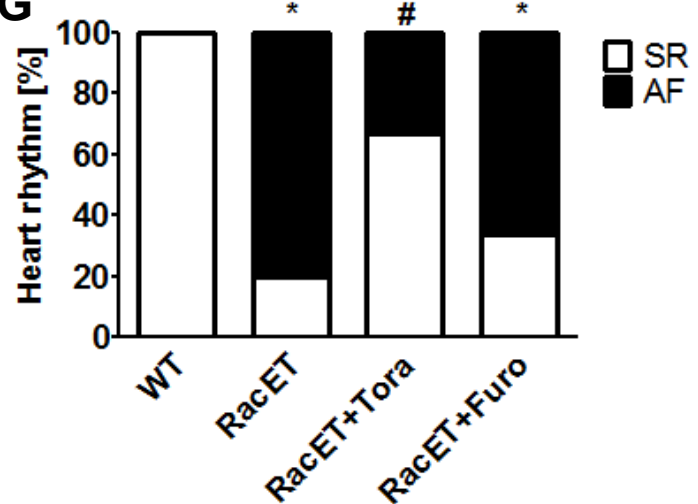


Table: Characteristics of FVBN, RacET, RacET-Torasemid and RacET-Furosemid					
n=10	FVBN	RacET	RacET-Torasemid	RacET-Furosemid	p Value
Mouse (g)	27.12 ± 0.55	25.81 ± 1.00	2.10 ± 1.5	25.12 ± 0.70	NS
Tibia (mm)	18.60 ± 0.24	18.55 ± 0.21	18.53 ± 0.22	17.52 ± 0.11*†•	<0.05
Heart (mg)	132.3 ± 4.23	216.15 ± 26.34*	191.12 ± 79.48	155.68 ± 8.51	<0.05
Atria (mg)	18.48 ± 3.25	41.41 ± 8.91	24.53 ± 5.30	34.11 ± 6.57	NS
LA (mg)	2.00 ± 0.07	6.91 ± 1.12	4.01 ± 0.67	5.32 ± 0.66	NS
RA (mg)	7.2 ± 1.80	14.78 ± 2.72	15.15 ± 6.21	28.79 ± 6.28	NS
LV (mg)	80.42 ± 5.63	78.91 ± 10.36	95.9 ± 8.41	77.45 ± 5.00	NS
RV (mg)	15.86 ± 1.66	21.92 ± 4.04	69.61 ± 32.38	20.58 ± 0.98	NS
Atria (mg)/heart (mg)	0.14 ± 0.03	0.17 ± 0.02	0.13 ± 0.03	0.21 ± 0.03	NS
Heart (mg)/tibia (mm)	7.11 ± 0.21	11.76 ± 1.52*	13.72 ± 5.19	8.89 ± 0.48	<0.05
Atria (mg)/tibia (mm)	0.99 ± 0.17	2.26 ± 0.5	1.31 ± 0.33	1.95 ± 0.38	NS
Atria (mg)/heart (mg)/tibia (mm)	2.66 ± 0.56	3.21 ± 0.32	2.65 ± 0.72	3.44 ± 0.49	NS
Liver wet (mg)	1151.45 ± 42.05	1240.66 ± 128.47	1426 ± 157.34	1211.05 ± 32.38	NS
Liver dry (mg)	395.35 ± 12.25	376.76 ± 33.05	440.75 ± 48.16	365.94 ± 9.29	NS
Liver wet (mg)-liver dry (mg)	756.1 ± 29.8	863.9 ± 96.16	985.25 ± 109.98	845.11 ± 23.92	NS
Lung wet (mg)	183.7 ± 55.8	143.02 ± 6.55	154.28 ± 6.96	190.26 ± 10.60†	<0.05
Lung dry (mg)	42.25 ± 10.55	30.52 ± 2.00	31.55 ± 3.59	37.37 ± 1.62	NS
Lung wet (mg)-lung dry (mg)	141.45 ± 45.25	112.5 ± 6.33	122.73 ± 7.60	121.12 ± 9.07†	NS
Hemodynamic: n=10	FVBN	RacET	RacET-Torasemid	RacET-Furosemid	p Value
Bp (systolic)	123.47 ± 5.23	117.39 ± 3.26	119.48 ± 3.87	113.73 ± 2.24	NS
Bp (diastolic)	110.2 ± 3.97	99.63 ± 3.67	100.26 ± 3.59	97.45 ± 2.48	NS
Pulse	595.95 ± 23	541.73 ± 15.3	517.93 ± 9.51*	576.68 ± 7.94•	<0.05
Urine:n=5	FVBN	RacET	RacET-Torasemid	RacET-Furosemid	p Value
g urine/24 h	0.46 ± 0.10	0.85 ± 0.22	1.12 ± 0.28	1.25 ± 0.13*	0.035
spez. weight g/l	1.03 ± 0.01	1.02 ± 0.01	1.02 ± 0.02	1.03 ± 0.01	NS
ml urine/24 h	0.45 ± 0.10	0.84 ± 0.23	1.1 ± 0.27	1.12 ± 0.16	NS
CU µg/l	56.06 ± 19.02	32.52 ± 10.66	15.03 ± 2.54	143.07 ± 24.66•	<0.05
CU µg/24 h	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.10 ± 0.01*•	<0.05
Na mmol/l	38 ± 11.71	45 ± 23.72	19.67 ± 3.22	72.00 ± 10.53•	<0.05
Na mmol/24 h	0.017 ± 0.006	0.027 ± 0.01	0.018 ± 0.01	0.055 ± 0.01*†•	<0.044
K mmol/l	66.02 ± 20.87	62.42 ± 32.74	30.83 ± 7.76	198.20 ± 19.42•	<0.05
K mmol/24 h	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.14 ± 0.01*†•	<0.05
Ca mmol/l	3.32 ± 1.65	0.54 ± 0.20	0.56 ± 0.11	2.45 ± 0.39†•	<0.05
Ca mmol/24 h	0.00143 ± 0.0007	0.0003 2± 0.0007	0.00049 ± 0.00007	0.001780 ± 0.00033	<0.05
Serum: n=5	FVBN	RacET	RacET-Torasemid	RacET-Furosemid	p Value
Ca mmol/l	2.21 ± 0.24	2.06 ± 0.05	2.02 ± 0.03	2.1 ± 0.04	NS
Crea µmol/l	0.28 ± 0.02	0.3 ± 0.01	0.28 ± 0.01	0.35 ± 0.01*†•	<0.005
Na mmol/l	146.66 ± 1.82	150.94 ± 1.03	147.6 ± 0.69	140.94 ± 1.99†	<0.05
K mmol/l	6.75 ± 0.92	6.38 ± 0.63	5.25 ± 0.21	7.01 ± 0.85	NS
Cl mmol/l	116.02 ± 0.88	114.84 ± 0.73	108.3 ± 0.93*†	114.96 ± 1.96•	<0.045
Echo: n= 6-10	FVBN	RacET	RacET-Torasemid	RacET-Furosemid	p Value
LA (mm)	1.98 ± 0.19	3.74 ± 0.45*	2.12 ± 0.19†	2.441 ± 0.11†	<0.05
IVSd (mm)	0.78 ± 0.03	0.76 ± 0.03	0.64 ± 0.02*	0.75 ± 0.06	<0.05
LVEDd (mm)	4.21 ± 0.1	4.38 ± 0.19	3.82 ± 0.14	3.59 ± 0.14	<0.05
LVPWd (mm)	0.83 ± 0.04	0.85 ± 0.05	0.72 ± 0.03	0.89 ± 0.07	NS
IVSs (mm)	1.03 ± 0.05	0.93 ± 0.02	0.85 ± 0.02	1.04 ± 0.06	NS
LVESd (mm)	3.13 ± 0.13	3.33 ± 0.23	2.72 ± 0.14	2.56 ± 0.21	NS
LVPWs (mm)	1.01 ± 0.07	1.05 ± 0.06	0.89 ± 0.04*†	1.16 ± 0.11	<0.05
FS (%)	28.83 ± 2.23	22.98 ± 1.33*	29.19 ± 1.45†	29.13 ± 3.68†	<0.05
* Significant versus FVBN	† Significant versus RacET	• Significant versus Tora			

