Abstract—We investigated whether or not p38 mitogen-activated protein kinase inhibition ameliorates angiotensin II–induced target organ damage. We used double transgenic rats harboring both human renin and angiotensinogen genes (dTGRs). dTGR, with or without p38 inhibitor (BIRB796; 30 mg/kg per day in the diet), and nontransgenic Sprague–Dawley rats were studied in 2 protocols. In protocol 1 (week 7), systolic blood pressure of untreated dTGRs was 204±4 mm Hg, but partially reduced after BIRB796 treatment (166±7 mm Hg), whereas Sprague–Dawley rats were normotensive. The cardiac hypertrophy index was unchanged in untreated and BIRB796-treated dTGRs. The β-myosin heavy chain expression of BIRB796-treated hearts was significantly lower in BIRB796 compared with dTGRs, indicating a delayed switch to the fetal isoform. BIRB796 treatment significantly reduced cardiac fibrosis, connective tissue growth factor, tumor necrosis factor-α, interleukin-6, and macrophage infiltration. Albuminuria was not reduced in BIRB796-treated dTGRs. Tubular and glomerular damage with tumor necrosis factor-α expression was unaltered, although serum creatinine and cystatin C were normalized. Renal macrophage infiltration, fibrosis, and vessel damage were reduced. In protocol 2 (week 8), we focused on mortality and arrhythmogenic electrical remodeling. Mortality of untreated dTGRs was 100% but was reduced to 10% in the BIRB796 group. Cardiac magnetic field mapping showed prolongation of depolarization and repolarization in untreated dTGRs compared with Sprague–Dawley rats with a partial reduction by BIRB796. Programmed electrical stimulation elicited ventricular tachycardias in 81% of untreated dTGRs but only in 48% of BIRB796-treated dTGRs. In conclusion, BIRB796 improved survival, target organ damage, and arrhythmogenic potential in angiotensin II–induced target organ damage. (Hypertension. 2007; 49:481-489.)

Key Words: angiotensin II ■ p38 ■ electrical remodeling ■ cardiac and renal damage

Mitogen-activated protein kinase (MAPK) p38 is a 4-isofrom (p38α, p38β, p38γ, and p38δ) serine/threonine kinase originally isolated from lipopolysaccharide-stimulated monocytes. P38 kinase pathway activation results in phosphorylation of transcription factors affecting cell division, apoptosis, and invasiveness of inflammatory cells. P38α is involved in the biosynthesis of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and other important inflammatory cytokines that represent a convergence point for many proinflammatory signaling processes. P38α/β inhibitors were developed as a potential treatment for inflammatory diseases. However, p38 is also activated by cathecholamines, angiotensin (Ang) II, endothelin-1, hypoxia, and vascular wall stress. Ang II activates p38 in various cell types, including cardiomyocytes, vascular smooth muscle cells, mesangial cells, and monocytes. P38 MAPK and extracellular signal regulated kinase (ERK) 1/2 activation depend on the epidermal growth factor receptor. P38 MAPK, ERK, and tyrosine kinase signaling also play a role in pathogenesis of cardiovascular disease. p38 MAPK inhibition may, therefore, be of interest for cardiovascular diseases, including electrical remodeling. We tested this notion in an Ang II–dependent double transgenic rat (dTGR) model harboring the human angiotensinogen and renin genes. The rats develop hypertension, cardiac hypertrophy, proteinuria, and renal failure and die abruptly between age 7 to 8 weeks. Inflammation and innate and acquired immunity are important in the dTGR model. Others, as well as our laboratories, showed earlier that strategies directed at nuclear factor κB, TNF-α, corticosteroids, aldosterone, antioxidants, and antilymphocyte treatments all ameliorate target organ damage that primarily involves the heart, kidneys, and vessels. We investigated MAPK p38 signaling in our model by using a novel p38
MAPK-α and β inhibitor (BIRB796). Because transgenic rats die suddenly, we also entertained the possibility of an arrhythmogenic death in the animals and investigated electrical remodeling.

Methods

Experimental Design

We studied male transgenic dTGRs (RCC Ltd) and age-matched nontransgenic Sprague–Dawley (SD) rats (MDC).17–20,23,25,26 Local authorities approved the studies, and American Physiological Society guidelines for animal care were followed. We performed 2 different protocols. In protocol 2, untreated dTGR (n = 15), dTGR + BIRB796 (30 mg/kg per day in the diet for 3 weeks; n = 11), and SD (n = 8 each group) rats were analyzed. The structure of BIRB796 and kinase selectivity is shown in Figure IA (available online at http://hyper.ahajournals.org). Systolic blood pressure was measured weekly by tail cuff. Twenty-four–hour urine samples were collected in metabolic cages from weeks 5 to 7. Serum was collected at week 7. Serum creatinine and cystatin C were measured by clinical routine assays. Urinary rat albumin was determined by enzyme-linked immunosorbent assay (CellTrend). The aim of protocol 2 was to focus on electrophysiological alterations and mortality. Untreated dTGR (n = 10), dTGR + BIRB796 (n = 10), and SD (n = 10) rats were studied up to week 8. Cardiac magnetic field mapping (CMFM) was performed at week 7 under isoflurane anesthesia. Echocardiography was performed as described earlier.18

Cardiac Magnetic Field Mapping and In Vivo Electrophysiology

Magnetic fields of the rat heart were recorded over the anterior chest. Details are given in the online supplement. Programmed electrical stimulation was performed to test for the inducibility of ventricular arrhythmias. Details are given in the online supplement.

Immunohistochemistry

Ice-cold acetone-fixed cryosections (6 μm) were stained by immunofluorescence or alkaline phosphatase-anti-alkaline phosphatase techniques as described earlier.19,20 Details are given in the online supplement.

Quantitative TaqMan RT-PCR

RNA isolation and TaqMan RT-PCR were performed as described earlier.21 We analyzed left ventricular tissue for β-myosin heavy chain, connective tissue growth factor, and IL-6. Each sample was in triplicate. For quantification, the target sequences were normalized in relation to the 36B4 product. Biotec synthesized the primers. The sequences are available on request.

Statistics

Data are presented as mean ± SEM. Statistically significant differences in mean values were tested by ANOVA, and blood pressure and albuminuria by repeated-measures ANOVA and the Scheffé t test. A value of P < 0.05 was considered statistically significant. The data were analyzed using Statview statistical software.

Results

p38 MAPK Inhibition Reduces Blood Pressure Without Reduction of Cardiac Hypertrophy but Affected Fetal β-Myosin Heavy Chain Expression and Proliferation of Cardiomyocytes

Systolic blood pressure increased progressively in untreated dTGRs from 155 ± 3 mm Hg in week 5 to 204 ± 4 mm Hg in week 7. The BIRB796 treatment slightly reduced blood pressure (166 ± 7 mm Hg at week 7; P < 0.05; Figure 1A), whereas SD rats were normotensive (123 ± 3 mm Hg). Despite the reduction in blood pressure, untreated and BIRB796-treated dTGRs had similar heart weight (data not shown) and cardiac hypertrophy indices (heart-to-tibia ratio), which were significantly higher compared with nontransgenic SD rats (310 ± 6 versus 307 ± 6 versus 206 ± 5 mg/cm, respectively; P < 0.05; Figure 1B). Echocardiography confirmed the results of cardiac hypertrophy. Total wall thickness (expressed as sum of septum + left ventricular posterior wall) of dTGRs was 3.3 ± 0.1 mm, 3.8 ± 0.1 mm for BIRB796, and 1.8 ± 0.05 mm for SD rats (Figure 1C). In contrast to untreated dTGRs, BIRB796-treated hearts showed a significantly lower expression of the fetal β-myosin heavy chain isoform indicating a different pattern of cardiac hypertrophy (Figure 1D). We next analyzed cardiomyocyte proliferation. The number of Ki-67-positive cells in the heart (Figure 1E) was significantly higher in untreated dTGRs compared with SD rats. BIRB796 treatment resulted in a further significant increase of proliferating cardiomyocytes and fibroblasts. Localization of Ki-67 positive nuclei is shown in Figure II.

BIRB796 Reduces p38 MAPK and ERK 1/2 Phosphorylation, Fibrosis, Cytokine Expression, and Macrophage Infiltration in the Heart

We investigated whether or not BIRB796 treatment affected MAPK phosphorylation in vivo. Untreated dTGRs show increased p-p38 MAPK and p-ERK 1/2 expression, which was significantly reduced by BIRB796 (Figure 2A). Untreated dTGRs showed marked signs of fibrosis. Collagen I was expressed predominantly in the interstitium and perivascularly (data not shown). Cardiac fibronectin immunoreactivity was observed predominantly in the interstitium and perivascularly (data not shown). BIRB796 treatment significantly reduced TNF-α (Figure 2A), connective tissue growth factor, and IL-6 expression (Figure 2B). We next analyzed cardiac connective tissue growth factor (Figure 2C) and IL-6 (Figure 2D) gene expression. Both genes were significantly reduced in the left ventricle after BIRB796 treatment. Cardiac macrophage and monocyte infiltration (ED-1+ cells) was increased in untreated dTGRs, significantly reduced by BIRB796, but still elevated compared with SD rats (Figure IIIA).

p38 MAPK Inhibition Does Not Affect Albuminuria but Normalizes Serum Creatinine and Cystatin C and Reduces Macrophage Infiltration

Surprisingly, BIRB796 treatment did not ameliorate albuminuria compared with untreated dTGRs (30 ± 5 versus 33 ± 5 mg/day, respectively; Figure 3A). Both groups showed a significantly increased urinary albumin excretion compared with SD (0.1 ± 0.02 mg/day). In contrast, only untreated dTGRs had an elevated serum creatinine (Figure 3B) and altered cystatin C (Figure 3C), whereas BIRB796 treatment normalized creatinine and cystatin C to SD levels. Monocyte/macrophage infiltration in the kidney was significantly increased in untreated dTGRs, reduced by BIRB796, but still elevated compared with SD (Figure IIIIB). The effect of BIRB796 on p38 MAPK in the kidney is shown in Figure IVA-C.
p38 MAPK Inhibition Reduces Renal Interstitial but Not Glomerular Fibrosis and Prevented Vascular but Not Tubular and Glomerular Damage

Renal histology (Masson trichrome staining) revealed that untreated dTGRs had increased perivascular, interstitial, and glomerular matrix deposition; vessel with hypertrophied media; and damaged tubules and glomeruli (Figure 3D). Tubules were often filled with protein in BIRB796-treated rats even more frequently compared with untreated dTGRs (Figure 3D). Chronic BIRB796 treatment resulted in a significantly reduced interstitial matrix formation and a reduction of vessel damage (Figure 3D). In contrast, tubules and glomeruli were still damaged (Figure 3D). Interstitial fibronectin expression was significantly reduced by BIRB796 (Figure 3E), whereas glomeruli from BIRB796-treated rats showed an unchanged collagen III expression compared with dTGRs (Figure 3F). Renal TNF-α immunoreactivity was increased in damaged tubules, glomeruli, and vessels of untreated dTGRs. However, BIRB796 reduced vascular TNF-α (see Figure 3D, inset) but had no effect on glomerular and tubular expression (Figure 3G).

Figure 1. A, BIRB796 reduced blood pressure only slightly at week 7. B and C, Untreated and BIRB796-treated dTGRs both showed increased cardiac hypertrophy (heart weight/tibia ratio) and total wall thickness (expressed as septum+/left ventricular wall diameter) compared with SD. D, BIRB796 treatment reduced the fetal β-myosin heavy chain isoform. E, Untreated dTGR hearts have more Ki-67+ cells compared with SD, whereas BIRB796 treatment further increased it by 20% compared with untreated dTGRs. BIRB796 results are mean±SEM. *P<0.05 vs untreated dTGR, #P<0.05 vs dTGR+BIRB796.

p38 MAPK Inhibition Improves Electrophysiological Alterations

We next focused on electrophysiological alterations and mortality by analyzing CMFM recordings. Untreated dTGRs had a significantly prolonged QRS duration and QT interval compared with SD controls. BIRB796 treatment resulted in a partial but significant reversal of this pattern (Table I). We also analyzed parameters reflecting the inhomogeneity of depolarization (mean inhomogeneity index for CMFM distribution during QRS interval) and repolarization (peak of the T-wave [Tpeak]-end of the T-wave [Tend] interval and Tpeak-Dispersion), which were significantly increased in untreated dTGRs compared with SD controls and partially restored by BIRB796 (Table I). Indicators of regional/spatial differences in the process of repolarization were significantly increased in dTGRs compared with SD controls (Table I). Inhomogeneity of repolarization was improved by BIRB796 treatment (Table I).

CMFM distribution for QRS and ST-T wave is shown in Figure VA and VB. Untreated dTGRs show an increase in field strength and duration of the QRS compared with SD controls. BIRB796 treatment ameliorated these changes (Figure VA). ST-T wave maps visualize prolongation of repolarization in untreated dTGRs and were partially ameliorated by BIRB796 treatment. These results were confirmed by a reorientation of the cardiac magnetic field in the end of the T wave (Table I).

“Butterfly” plots are CMFM waveform composites from all of the animals per group (Figure VIA). Inserted magnification show that the dispersion of the T wave peak was...
increased in dTGRs compared with controls. BIRB796 treatment ameliorated the “butterfly” pattern (Figure VIA) and significantly reduced the T wave dispersion (Figure VIB). Untreated dTGRs showed a prolongation of repolarization by 15 ms and a flattening of the peak of T wave, which was partially corrected by BIRB796 (Figure VIC).

p38 MAPK Inhibition Reduces Arrhythmias by Programmed Electrical Stimulation
We next analyzed whether untreated dTGRs are prone to ventricular arrhythmia induction and whether BIRB796 treatment affects arrhythmia induction. Programmed electrical stimulation showed a high nonsustained and sustained ventricular tachycardia induction rate in untreated dTGRs (81%, Figure 4A). In SD controls, the same protocol never initiated arrhythmias. The arrhythmia induction was significantly reduced in BIRB796-treated dTGRs (48%). In addition, the mean cycle lengths of induced ventricular tachycardias were significantly shorter in untreated compared with BIRB796-treated dTGRs (Figure 4B). Representative recordings of performed electrophysiological studies are shown (Figure 4C).

p38 MAPK Inhibition Reduces Mortality
Finally, chronic BIRB796 treatment significantly reduced mortality (Figure 5). Although mortality in untreated dTGRs was 100%, only 1 (10%) of 10 BIRB796-treated dTGRs died at the last day of the study (day 56). SD controls had no mortality by week 8.

Discussion
Three major findings of the present study are that chronic p38 MAPK inhibition reduced mortality in Ang II–induced end-organ damage, that p38 MAPK inhibition showed a highly cell type–specific action leading to protection of the heart and vessels, and that renal tubular and glomerular damage were less ameliorated, although glomular filtration rate was improved. Furthermore, p38 MAPK inhibition improved electrophysiological alterations and reduced arrhythmias by programmed electrical stimulation.

We found that p38 MAPK inhibition with BIRB796 reduced cardiac fibrosis, connective tissue growth factor, TNF-α, and IL-6 expression, as well as vascular damage and macrophage infiltration. Although BIRB796 treatment did not alter the heart weight/tibia ratio and total wall thickness,
Figure 3. A, Albuminuria was not different between untreated and BIRB796-treated dTGRs, although serum creatinine (B) and cystatin C (C) were normal. D, Masson trichrome staining showed increased matrix deposition, vessel with hypertrophied media, damaged tubules, and glomeruli in untreated dTGRs. BIRB796 reduced matrix formation and vessel damage but not glomerular and tubular damage. Tubules were often filled with protein, indicated by *. Inset shows a higher magnification of the vessel indicated by the arrow. E, Renal fibronectin was partially reduced by BIRB796. F, BIRB796 had no effect on glomerular collagen III deposition. G, TNF-α was expressed in tubules and glomeruli of untreated and BIRB796-treated dTGRs. In contrast, BIRB796 reduced vascular expression. Semiquantitative scorings are given as insets.
p38 MAPK inhibition reduced β-myosin heavy chain expression, suggesting a less pathological and more physiological hypertrophy in BIRB796-treated dTGR hearts. p38 MAPK-inhibited hearts also showed an improved electrical remodeling and a decreased susceptibility to ventricular tachycardia induction. Although BIRB796 treatment did not reduce albuminuria, serum creatinine did not increase in BIRB796-treated dTGRs. Visible glomerular and tubular damage was present in BIRB796-treated dTGRs. Nevertheless, p38 MAPK inhibition reduced renal matrix formation and macrophage infiltration, as well as vascular damage. We believe that most of the effects are mediated via p38 α/β MAPK. Nevertheless, we cannot completely exclude the role of c-Jun NH2-terminal kinase (JNK)-2. In vitro, BIRB796 inhibits JNK-2 with an IC50 of 2 nM, whereas BIRB796 has no effect on JNK-1. Nevertheless, it could well be that, in vivo, our chronic BIRB796 treatment resulted in drug levels sufficient to inhibit JNK-2.

We also found that blood pressure was partially reduced compared with untreated dTGRs. Nonetheless, hypertension persisted at a level of >165 mm Hg. We do not believe that blood pressure reduction alone was a major cause for the improvement of target organ damage.19,25 Several points suggest a blood pressure-independent effect. First, despite a blood pressure decrease of ~40 mm Hg, cardiac mass/hypertrophy was similar in untreated and BIRB796-treated dTGRs, but the nature of the cardiac phenotype was different. In general, cardiac mass/hypertrophy clearly depends on afterload. Therefore, we could well imagine that a reduction of 40 mm Hg afterload might have facilitated to reverse the...
pathophysiological hypertrophy in untreated dTGRs to a physiological hypertrophy observed in BIRB796-treated dTGRs. Nonetheless, load, per se, cannot be the sole reason. BIRB796-treated dTGRs were still hypertensive but showed almost normalized matrix and cytokine expression, as well as reduced cell infiltration. We did not include a parallel blood pressure control group (eg, hydralazine treatment) in this study. However, earlier we performed several studies that separated Ang II–related from blood pressure–related effects in our model.25 We treated dTGRs treated with triple therapy (hydralazine, hydrochlorothiazide, and reserpine). This treatment merely delayed the course of the disease by 1 week. In other experiments, we treated dTGRs with dexamethasone. Dexamethasone-treated dTGRs were severely hypertensive (blood pressure levels >200 mm Hg). Nevertheless, dexamethasone reduced mortality to 0 with significantly improved target organ damage.19

In an earlier study, we found that untreated dTGRs exhibited an α/β-myosin heavy chain switch to the fetal isoform, as we confirm here.28 In that study, atrial natriuretic peptide precursor, a marker of cardiac hypertrophy, genes of the cell cycle (cyclin D3, cyclin D2, and cyclin D), heat shock proteins (HSP70 and HSP27), and genes of the MAPK pathway, namely, heparin-binding epidermal growth factor (epidermal growth factor–like) growth factor, p38 MAPK, c-fos, junB, fra, and c-myc, were all significantly increased in the left ventricles of terminally ill dTGRs. Interestingly, heat shock proteins themselves activate the p38 pathway. These findings are relevant to the present results, because BIRB796 treatment ameliorated target organ damage and reduced mortality significantly. Whether the heart can grow by multiplication of myocytes has been controversial over the decades. Recent data indicate that the adult heart has a subpopulation of myocytes that is not terminally differentiated. These myocytes evidently reentered the cell cycle and underwent nuclear mitotic division. Beltrami et al29 provided convincing proof of myocyte replication in the failing human heart and showed that this form of cell growth could compensate for exhaustion of myocyte hypertrophy. Recently, Engel et al30 demonstrated that p38 MAPK inhibition promotes cardiomyocyte cytokinesis. Our data show that untreated hypertrophied dTGR hearts have a higher number of Ki-67–positive cardiomyocytes compared with nontransgenic hearts. Interestingly, BIRB796 treatment further increased myocyte proliferation by 20%. It is tempting to speculate that increased cardiomyocyte proliferation might have contributed to tissue repair in our model.

The p38 MAPK module is activated by a plethora of other MAPK kinases in response to numerous physical and chemical stresses, including Ang II, aldosterone, various cytokines, oxidative stress, hypoxia, and ischemia.1,31–34 Recently, we found that blockade of aldosterone synthase ameliorates fibrosis and inflammation in our model.23 Possibly, aldosterone-p38 signaling might have contributed to the pathogenesis of organ damage in our dTGR model. Interestingly, various in vivo studies in transgenic rats over-expressing the mouse ren-2 gene, another model of Ang II–induced target organ damage, demonstrated that not only p38 MAPK but also ERK 1/2 and JNK signaling are involved in the pathogenesis.8,14,16 In our model, we found that BIRB796 also reduced cardiac ERK 1/2 phosphorylation. We believe that this result might have been an indirect phenomenon because of improved cardiac function with reduced cell infiltration and cytokine production. Altogether the findings in both transgenic models suggest that MAPK signaling plays an important role in Ang II–induced target organ damage.

Innate and acquired immunity also plays an important role in our dTGR model. Untreated dTGRs showed elevated TNF-α, IL-6, and C-reactive protein levels.18–20,26 The p38α kinase inhibitor BIRB796 blocks the biosynthesis of TNF-α, C-reactive protein, IL-1β, and other important inflammatory cytokines in humans.35 In vivo, the proinflammatory situation is not only determined by cytokine production and release of immune cells but also by other cell types, like smooth muscle cells, tubular cells, and glomerular cells. BIRB796 treatment led to an organ and cell type–specific reduction of inflammatory actions. Although BIRB796 reduced IL-6 and TNF-α expression in the heart and macrophage infiltration in heart and kidney, the drug only reduced vascular but not tubular and glomerular TNF-α expression in the kidney. Shao et al reported that Ang II–infused rats showed an increase in the T-helper 1 cytokine interferon-γ.36 Rincon et al37 reported that the p38 MAPK pathway is necessary for interferon-γ production by T-helper 1 effector cells without affecting the T-helper 2 response. P38 is also activated in macrophages, neutrophils, and T cells, where it participates in respiratory burst activity, chemotaxis, granular exocytosis, adherence, and apoptosis. P38 also mediates immune responses by stabilizing specific cellular mRNAs involved in these processes. The precise functions of p38 are only now beginning to emerge; however, p38 has been shown to phosphorylate several cellular targets, including cytosolic phospholipase A2, the microtubule-associated protein Tau, and the transcription factors ATF1 and -2, MEF2A, Sap-1, Elk-1, nuclear factor κB, Ets-1, and p53. There are numerous p38 targets that could have influenced the course of target organ damage in our model. Nuclear factor κB is a particularly attractive target that received our attention in earlier studies.18,26 We have not explored the pathways involved. However, other investigators have drawn attention to interactions among Ang II, reactive oxygen species, and p38 MAPK31,32,34 that could very well be implicated here. In vitro, but also in vivo, Ang II also promotes p38 MAPK activation via the epidermal growth factor receptor and platelet-derived growth factor receptor β.14,16

Among our major focal points was electrical remodeling. The term “remodeling” is generally used morphologically. Wijffels et al18 defined the term electrical remodeling as the occurrence of cardiac arrhythmias because of acquired changes in cardiac structure or function. Increased fibrosis is an important factor that affects electrical conduction leading to an increased susceptibility to arrhythmias.39 Inflammation also promotes electrical remodeling.40 The morphological alterations in the heart of our dTGR rats have been addressed in an earlier study.28 A part of our hypothesis in the current study involved the cause of death in these animals. We have not addressed that issue directly. Continuous ECG telemetry in a large number of animals would be necessary. Nevertheless, we have introduced a preliminary approach to address...
this issue. Our presumption requires verification; however, the results presented here suggest that the electrical remodeling process can be influenced by an anti-inflammatory intervention mediated by a signaling molecule, namely, p38.

Perspectives
Our findings underscore the importance of inflammatory and electrophysiological pathways in target organ damage. Views on p38 have invariably considered anti-inflammatory applications. Cardiovascular disease is not generally considered to be an inflammatory or immune disorder. Our model and the data presented here support the therapeutic perspective of anti-inflammatory and antiarrhythmic approaches to cardiovascular disease.

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Disclosures
F.C.L. has served as an advisor for Boehringer-Ingelheim Pharmaceuticals. The remaining authors report no conflicts.

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p38 Mitogen-Activated Protein Kinase Inhibition Ameliorates Angiotensin II–Induced Target Organ Damage

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Methods on-line supplement

Immunohistochemistry

The sections were incubated with the following monoclonal antibodies: anti-ED-1, which detects monocytes and macrophages, anti-Ki-67, a nuclear cell proliferation-associated antigen expressed in all active stages of the cell cycle (Dianova, Germany), and polyclonal antibodies rabbit anti-p38 MAPK (Thr180/ Tyr182; Cell Signaling, Germany), anti-ERK1/2 (Thr202/Tyr204; Cell Signaling, Germany) anti-fibronectin (Paesel, Germany), anti-collagen I (Southern Biothechnology, USA), anti-collagen III (RDI Research Diagnostics, USA) and goat anti-TNF-α (Santa-Cruz, Germany). For quantification, sections were analyzed with a Zeiss Axioplan-2 microscope (Carl Zeiss, Germany) and AxioVision multi channel image processing system (Carl Zeiss, Germany). Semiquantitative scoring of infiltrated monocytes/macrophages (ED+ cells) Ki-67+ cells (MIB-5), in 15 different cortical kidney areas and 15 cardiac areas (n=6 per group), was performed using computerized cell count program (KS 300 3.0, Zeiss, Germany) with samples examined blind. Collagen I, III and fibronectin expressions were scored in arbitrary units (0-5+) based on the staining intensity.

Cardiac Magnetic Field Mapping and in vivo Electrophysiology

Magnetic fields (MF) of the rat heart were recorded over anterior chest wall using a magnetic measurement system (Cryoton Ltd.) with seven MF sensors based on low temperature superconducting quantum interference devices (SQUIDs) coupled with axial second-order gradiometers (baseline 55 mm, pickup coil diameter 20 mm). ECG lead II was simultaneously recorded for CMFM time processing (30 seconds, 1000 Hz sampling rate). Programmed electrical stimulation was performed to test for the inducibility of ventricular arrhythmias.

Programmed electrical stimulation
Programmed electrical stimulation was performed to test for the inducibility of ventricular arrhythmias. We used a digital electrophysiology (EP) lab (EP Tracer, CardioTek, Netherlands). During inhalation anesthesia with isoflorane animals’ body temperature was kept constant by using a warming light. Two small octapolar EP catheters (CIBer mouse cath, NuMed Inc.) were placed in the right atrial and left ventricular cavity via the right jugular vein and the right carotid artery. Programmed ventricular stimulation was performed using a standardized protocol which included trains of 10 basal stimuli (S1) followed by up to 3 extra stimuli (S2-S4), delivered with a coupling interval decreasing in steps of 5 ms until ventricular refractory was reached. Occurrence and duration of inducible arrhythmias were documented. Only reproducible arrhythmias up from 3 beats were considered. The number of animals with inducible arrhythmias in protocols with basal cycle lengths of 150, 120, 100, and 80 ms as well as the mean cycle length of induced ventricular arrhythmias was compared between the groups.

**Results on-line supplement**

On-line supplement figure I in the supplement section, which demonstrates the structure of BIRB796 (panel A), a scheme of MAPK activation pathway as well as selectivity of BIRB796 for certain kinases (panel B). We performed co-staining with desmin and Ki-67 and present a representative picture of an untreated dTGR heart section in supplement figure II. Our results demonstrate that nuclei of cardiomyocytes (indicated by the arrows) as well as fibroblasts (indicated by the asterisk) were stained for Ki-67.

Monocyte/macrophage infiltration in the heart and kidney shows a reduced infiltration in BIRB796-treated dTGR compared to untreated dTGR (on-line supplement figure IIIA-B). We performed phospho-p38 MAPK immunostainings to evaluate the role of BIRB796 in the kidney. Scoring analysis revealed that BIRB796 treatment resulted in a slightly reduced
vascular and tubular p-p38 MAPK score compared to untreated dTGR, while there was no significant reduction in glomeruli (on-line supplement figure IVA-C).

CMFM distribution for QRS and ST-T wave is shown in Supplement Figure VA and VB. The maps are a composite of all animals per group and are analogous to relief contour maps. Red are signals directed towards the detectors, while blue are signals moving away from the detectors. The ring number reflects the magnetic field strength. Untreated dTGR show an increase in field strength and duration of the QRS compared to controls. The course of isolines, as well as the positions of positive (red) and negative (blue) extremes, were different in untreated dTGR particularly in the middle and second half of corresponding QRS complex (maps 10 to 18 ms). BIRB796 treatment ameliorated these changes (Supplement Fig. VA). ST-T wave maps visualize prolongation of repolarization in untreated dTGR indicated by a marked reduction in initial signal strength increase (first 10 ms after J point) and longer lasting field strength (number of isolines 40 and 50 ms after J point). Chronic BIRB796 treatment partially ameliorated the pathological pattern of untreated dTGR.

“Butterfly” plots are CMFM waveform composites from all animals per group (on-line supplement figure VIA). We focused on the difference in the ST-T wave segment with regard to the dispersion of the T wave peak in different sensor positions. Inserted magnification show that the dispersion of T wave peak was increased in dTGR compared to controls. BIRB796 treatment ameliorated the “butterfly” pattern (on-line supplement figure VIA) and significant reduced the T wave dispersion (on-line supplement figure VIB). The magnetic field range at RPeak was markedly increased in dTGR compared to SD. RPeak and J point of untreated dTGR were delayed compared to SD. Untreated dTGR showed a prolongation of repolarization by 15 ms and a flattening of the peak of T wave, which was partially corrected by BIRB796 (on-line supplement figure VIC).

Legends for on-line supplement figures
**Fig. I.** Panel A. Structure of BIRB796. Panel B, kinase selectivity of BIRB796.

**Fig. II.** Representative picture of an untreated dTGR heart stained with an anti-Ki-67 antibody. Red fluorescence represent positive nuclei. Arrows indicate myocytes; asterisk indicate fibroblasts. Anti-desmin staining (green) was used to visualize myocytes. The bar represents 50 µm.

**Fig. III.** Cardiac (panel A) and renal (panel B) macrophage infiltration.

**Fig. IV.** Renal phospho-p38 MAPK scoring. Glomerular (panel A), tubular (panel B) and glomerular p-p38 MAPK in dTGR, BIRB796-treated dTGR and SD rats. Results are mean±SEM. (* p<0.05 vs. dTGR+BIRB796, # p<0.05 vs. dTGR+BIRB796).

**Fig. V.** Magnetic field maps. Panel A. Depolarization in dTGR, BIRB796-treated dTGR and SD rats. Panel B. Repolarization in dTGR, BIRB796-treated dTGR and SD rats. Drug treatment ameliorated the patterns to resemble SD.

**Fig. VI.** **Panel A.** “Butterfly” plots with magnetic field strength. The QRS complex occurs within the first 25 ms. ST segment and T wave occupy the magnetic field strength between 25 and 110 ms. The inserts show the dispersion of T wave peaks in different sensor positions at a higher magnification, which is quantified in **Panel B.** The increased dispersion of the T wave peak in untreated dTGR was reduced by BIRB796. **Panel C** shows the magnetic field range as averaged curves for all 3 groups. Depolarization signals, corresponding to the ECG R wave, were prolonged and increased in strength in untreated dTGR. BIRB796 resulted in a slightly reduction of the pathological alterations. The prolonged repolarization of untreated dTGR, indicated by the pronounced delay of the ECG T peak, was ameliorated by BIRB796. Results are mean±SEM. (* p<0.05 vs. untreated dTGR, # p<0.05 vs. dTGR+BIRB796).
### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>dTGR</th>
<th>dTGR+BIRB796</th>
<th>SD</th>
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</thead>
<tbody>
<tr>
<td>IHi (QRS)</td>
<td>502±49*</td>
<td>456±43*</td>
<td>266±12</td>
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<tr>
<td>QRS (ms)</td>
<td>24.3±0.4*</td>
<td>22.6±0.3*,+</td>
<td>18.7±0.8</td>
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<tr>
<td>QT (ms)</td>
<td>119±14*</td>
<td>99±3*,+</td>
<td>82±2</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>116±5*</td>
<td>99±4*,+</td>
<td>82±2</td>
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<tr>
<td>QT&lt;sub&gt;Peak&lt;/sub&gt; (ms)</td>
<td>43±2*</td>
<td>35±2*,+</td>
<td>28±1</td>
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<tr>
<td>T&lt;sub&gt;Peak&lt;/sub&gt;-T&lt;sub&gt;End&lt;/sub&gt; (ms)</td>
<td>75±5*</td>
<td>64±3*,+</td>
<td>53±2</td>
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<tr>
<td>MF Angle T Begin (°)</td>
<td>147±14</td>
<td>142±9*</td>
<td>160±6</td>
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<tr>
<td>MF Angle T End (°)</td>
<td>177±5*</td>
<td>157±6+</td>
<td>159±5</td>
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mean±SEM; * p<0.05 vs. SD, +p<0.05 vs. dTGR
Supplement Fig. I

A

BIRB796

B

Kinase Selectivity of BIRB796

<table>
<thead>
<tr>
<th>KINASES</th>
<th>MAPKK</th>
<th>MAPK</th>
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<tr>
<td>SYK</td>
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<td>IKK2β</td>
<td>MKK6</td>
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<td>ERK</td>
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<td>EGFR</td>
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<td>PKC</td>
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<tr>
<td>PKC-α</td>
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<td>PKCb (I &amp; II)</td>
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<tr>
<td>PKC-γ</td>
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<td>RAF-1</td>
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<td>p59FYN</td>
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<td>p56LCK</td>
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> 10,000 FOLD

REGULATION OF TNFα, IL-6 etc.

STRESS RESPONSE FUNCTIONS

PROLIFERATIVE RESPONSE

MAPK FAMILY MEMBERS

ERK >10,000 FOLD

JNK2α2 1-2 FOLD
Supplement Fig. II
Supplement Fig. III

A

Cardiac Macrophage Infiltration

<table>
<thead>
<tr>
<th></th>
<th>dTGR</th>
<th>dTGR+BIRB796</th>
<th>SD</th>
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<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>10.5</td>
<td>6.0</td>
<td>2.0</td>
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<tr>
<td><strong>SD</strong></td>
<td>1.5</td>
<td>2.0</td>
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B

Renal Macrophage Infiltration

<table>
<thead>
<tr>
<th></th>
<th>dTGR</th>
<th>dTGR+BIRB796</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>25.0</td>
<td>10.5</td>
<td>5.0</td>
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<tr>
<td><strong>SD</strong></td>
<td>3.0</td>
<td>2.0</td>
<td>1.0</td>
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</tbody>
</table>
Supplement Fig. V

A  Magnetic Field Maps of Depolarization (QRS)

- $dTGR$
- $dTGR+ BIRB796$
- $SD$

Time (ms)

5  10  13  15  18  20

B  Magnetic Field Maps of Repolarization (JTEnd)

- $dTGR$
- $dTGR+ BIRB796$
- $SD$

Time (ms)

J +5  +10  +40  +50  +60
Supplement Fig. VI

A  Butterfly Plots

B  Peak of T-Wave Dispersion

C  Magnetic Field Range