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The treatment with soluble guanylate cyclase stimulator BAY41-8543 prevents malignant hypertension and associated organ damage

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Objective: Despite availability of an array of antihypertensive drugs, malignant hypertension remains a life-threatening condition, and new therapeutic strategies for the treatment of malignant hypertension and malignant hypertension-associated organ damage are needed. The aim of the present study was to assess the effects of nitric oxide (NO)-independent soluble guanylyl cyclase (sGC) stimulator on the course of malignant hypertension. The second aim was to investigate if the treatment with sodium-glucose cotransporter type 2 (SGLT2) inhibitor would augment the expected beneficial actions of the sGC stimulation on the course of malignant hypertension.

Methods: As a model of malignant hypertension, Ren-2 transgenic rats (TGR) treated with nonspecific NO synthase inhibitor (Nω-nitro- L-arginine methyl ester, L-NAME) was used. Blood pressure (BP) was monitored by radiotelemetry, and the treatment was started 3 days before administration of L-NAME.

Results: The treatment with sGC stimulator BAY 41-8543, alone or combined with SGLT2 inhibitor empagliflozin, abolished malignant hypertension-related mortality in TGR receiving L-NAME. These two treatment regimens also prevented BP increases after L-NAME administration in TGR, and even decreased BP below values observed in control TGR, and prevented cardiac dysfunction and malignant hypertension-related morbidity. The treatment with the SGLT2 inhibitor empagliflozin did not further augment the beneficial actions of sGC stimulator on the course of malignant hypertension-related mortality.

Conclusion: The treatment with NO-independent sGC stimulator displayed marked protective actions on the course of malignant hypertension-related mortality and malignant hypertension-related cardiac damage. This suggests that application of sGC stimulator could be a promising therapeutic means for the treatment of malignant hypertension.

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Keywords: malignant hypertension, renin–angiotensin system, sodium-glucose cotransporter type 2 inhibitor, soluble guanylyl cyclase stimulator

Abbreviations: ANG II, angiotensin II; cGMP, cyclic guanosine monophosphate; GDMT, guideline-directed medical therapy; GSI, glomerulosclerosis index; L-NAME, Nω-nitro-l-arginine methyl ester; LV, left ventricle; NO, nitric oxide; NOS, nitric oxide synthase inhibitor; RAS, renin–angiotensin system; sGC, soluble guanylyl cyclase; SGLT2, sodium-glucose cotransporter type 2; TGR, Ren-2 renin transgenic rats; TSI, tubulointerstitial injury

INTRODUCTION

Hypertension is the major independent risk factor of myocardial infarction, stroke, progression of chronic kidney disease and heart failure [1–4]. Malignant hypertension is the most severe and, if untreated, a fatal form of this disease: before introducing modern antihypertensive drugs, a 2-year survival rate was less than 20% [5–7]. As originally described by Volhard and Fahr [8], the hallmark of malignant hypertension is acute elevation of blood pressure (BP) to extremely high levels accompanied by acute microvascular damage affecting different organs,

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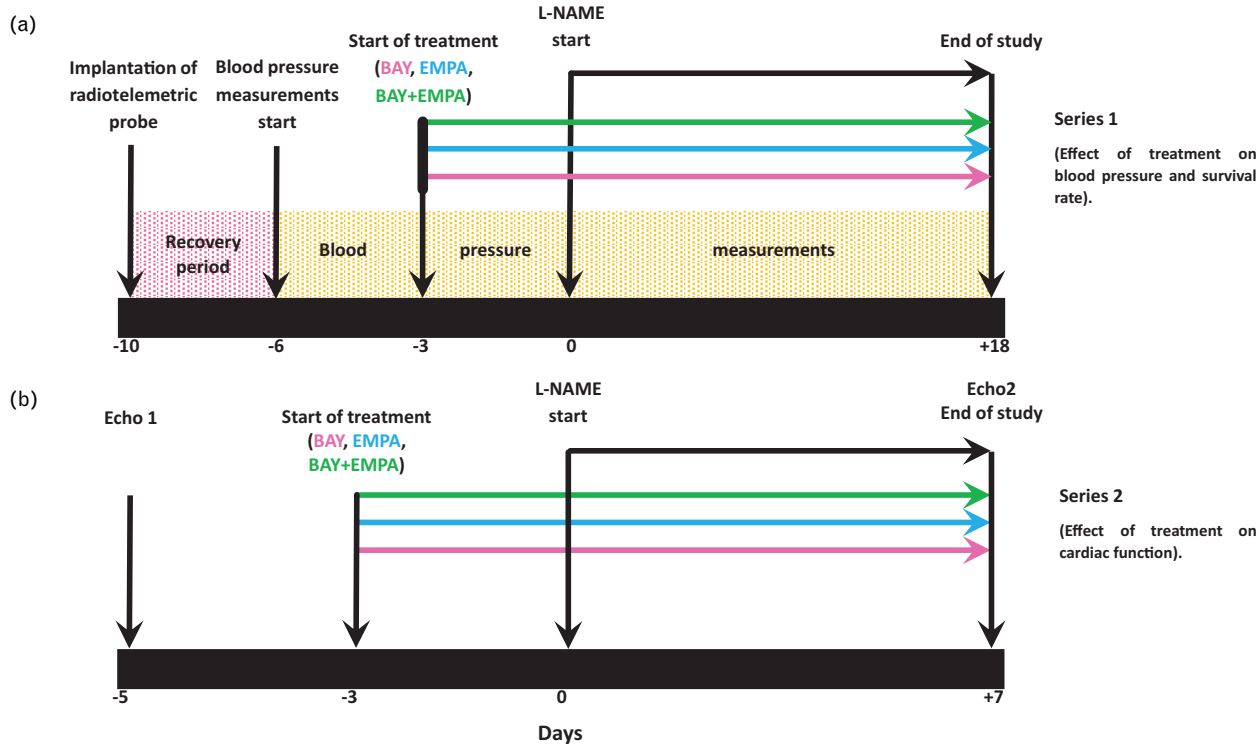

FIGURE 1 The experimental design of the whole study, delineating the time sequence of experimental manoeuvres and different treatment regimes. BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine), a stimulator of a soluble guanylyl cyclase; EMPA, empagliflozin, a sodium-glucose cotransport type 2 inhibitor; Echo, echocardiography assessment; L-NAME: Nω-nitro- L-arginine methyl ester, a nonspecific nitric oxide synthase inhibitor.

particularly the retina, brain, kidney and heart [9–14]. Even though malignant hypertension affects only 1–2% of all hypertensive patients [15] (the annual incidence is about 2 per 100 000 [2,3,7]), and full therapeutic armamentarium is available [as outlined in 'guideline-directed medical therapy' (GDMT)], malignant hypertension still remains a life-threatening condition, especially when associated with heart failure and kidney damage [2,7,16–19]. Therefore, new therapeutic strategies for the treatment of malignant hypertension and the associated organ damage, particularly heart failure, are still urgently needed to avoid cardiovascular and cardiorenal morbidity and mortality. Focused nonclinical studies employing experimental models of malignant hypertension should be performed to define new pharmacotherapeutic targets for prospective clinical research and development.

It is recalled that seminal studies from Laragh's group confirmed by other investigators [12,20–22] have proved that abnormal activation of the renin–angiotensin system (RAS) plays a critical role in the pathophysiology of malignant hypertension. It is also established that the nitric oxide (NO)/soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) complex plays a pivotal role in cardiovascular homeostasis and downregulation of NO/sGC/cGMP signaling, and is important in the pathophysiology of kidney damage and hypertension [23–26]. An animal model that combines increased RAS activity and decreased NO bioavailability seems to be promising to study pathophysiology of malignant hypertension and related organ damage and to evaluate new therapeutic approaches for the treatment this disease [27–30]. In this context, Ren-2 renin transgenic rat (TGR) [31,32], provides a model that combines endogenous activation of the RAS, an important pathophysiological component for the development of malignant hypertension [12,20–22], and hypertension. Both factors are critical for the development of organ damage, particularly for heart failure [33–37]. Therefore, TGR were used in the present study, and the rats were additionally treated with the nonspecific NO synthase (NOS) inhibitor [Nω-nitro-L-arginine methyl ester (L-NAME)], leading to NO deficiency, malignant hypertension and hypertension-induced heart failure and kidney damage, accompanied by increased morbidity and mortality [27–30,38,39].

As decreased activity of the NO/sGC/cGMP pathway (due to decrease in NO bioavailability), is one cause of malignant hypertension and associated organ damage, chronic NO-independent stimulation of sGC should attenuate the development of malignant hypertension and malignant hypertension-related end-organ damage. In fact, treatment with sGC stimulator vericiguat resulted in a positive outcome of a large clinical trial in patients with heart failure with reduced ejection fraction [40,41]. In addition, our recent experimental studies in TGR with high-output heart failure showing that the treatment with the sGC stimulator (BAY41-8543), which exhibits the same mode of action as vericiguat [25], effectively increased the survival rate as compared with untreated ACF TGR [42]. However, there is still a proportion of TGR with high-output heart failure, which could not be rescued by sGC stimulator treatment alone.

Therefore, the serendipitous discovery of the protective actions of sodium-glucose cotransport type 2 (SGLT2) inhibitors on the cardiorenal system and their effectivity in reducing the risk of cardiovascular and renal diseases in patients with heart failure with or without diabetes on the top of the standard GDMT [43–48] might be a highly effective treatment approach, especially in combination with a vasoactive sGC stimulators. However, the effects of SGLT2 inhibitors in the treatment of malignant hypertension and associated heart and kidney damage have not been so far evaluated in experimental studies. Moreover, a recent study by Salazar's group documented that SGLT2 inhibition potentiates the beneficial cardiovascular, renal and metabolic effects elicited by sGC stimulation in hypertensive rats with altered renal development exposed to prolonged exposure to high-fat diet [49]. These findings further support our notion that the NO-independent sGC stimulation as well as the treatment with SGLT2 inhibitors could be a novel therapeutic approach for malignant hypertension and associated organ damage.

Based on the above considerations, the first aim of the present study was to examine if the treatment with either sGC stimulator, BAY41-8543 alone or SGLT2 inhibitor, empagliflozin alone, could attenuate the development of malignant hypertension and the associated organ damage in TGR receiving with L-NAME.

The second aim of the present study was to find out if SGLT2 inhibitor empagliflozin will augment potential beneficial effects of BAY 41-8543 on the development of malignant hypertension and associated mortality and organ damage when given together.

METHODS

Ethical approval and animals

The study was performed in accordance with the guidelines and practices established by the Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine (IKEM), Prague, which accord with the European Convention on Animal Protection and Guidelines on Research Animal Use, and were approved by this committee and subsequently by the Ministry of Health of the Czech Republic (the decision number for this project is 14934/2022–5/OVZ. All animals used in the study were bred in IKEM, which is accredited by the Czech Association for Accreditation of Laboratory Animals. Experiments were performed in heterozygous TGR that were generated by breeding male homozygous TGR with female homozygous transgene-negative normotensive Hannover Sprague–Dawley rats. The study was carried out in compliance with the ARRIVE (Animals in Research: Reporting In vivo Experiments) guidelines [50].

Chemicals

L-NAME was given in drinking water at 100 mg/l, the dose that effectively blocks NOS activity, as documented in earlier studies, including ours [38,39,51].

sGC stimulator, BAY-41-8543 (2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine) is commonly used in preclinical trials as an analogue of vericiguat, with which it shares the identical mode of action and similar in-vitro and in-vivo profile [25]. This compound was given at a dose of 10 mg/kg/day (administered in the food), which was previously shown to be fully effective in this rat model [39]. We confirmed recently the effectiveness of its administration in the food in TGR [42].

SGLT2 inhibitor, empagliflozin showed positive outcomes in clinical heart failure trials and renal outcome trials [43–48]. This compound was given at 10 mg/kg/day (administrated in food), the dosage previously shown to be effective in TGR [52].

Detailed experimental design

The whole experimental design of the study, with presentation of the detailed time sequence of experimental maneuvers and treatment regimens is given in Fig. 1.

Series 1: effects of treatment with soluble guanylyl cyclase stimulator and sodium-glucose cotransporter type 2 inhibitor alone, or combined, on blood pressure and survival rate

In accordance with the recommendation for BP measurements in experimental animals [53,54], we employed a radiotelemetric system for direct BP measurements using the technique described in detail previously [38,42,55,56]. HD-S10 radiotelemetric probes (Data Science International, St. Paul, Minnesota, USA) were used for direct BP measurements and were implanted in male TGR of the initial age of 8 weeks, that is, identical age as used in Sharkovska's study [39] in which the TGR + L-NAME model was first characterized. Four days after implantation of radiotelemetric device, BP was recorded for 3 days, subsequently appropriate treatment regimens were initiated (i.e. treatment with BAY41-8543 or empaglifozin alone, or their combination), and BP was recorded for further 3 days. Then the treatment with L-NAME was started, and BP was recorded for 18 days. Body weight of all animals and daily food consumption were assessed continuously. The experimental protocol for this series is outlined in Fig. 1a. At the end of this series, all animals were killed by decapitation, and organ weights and tibia length were assessed. Plasma and whole kidney angiotensin II (ANG II) concentrations were measured in samples obtained from conscious decapitated rats (NB.: anesthesia substantially alters ANG II concentrations, particularly in ANG II-dependent hypertension) [59,60]. Plasma and kidney ANG II levels were measured by a competitive radioimmunoassay (RIA), using the commercially available RIA kit (Catalog number ED29051, IBL International, Hamburg, Germany), the method routinely employed in our laboratory [32,38,42,56–60].

The following experimental groups were examined in this series:

1. TGR + placebo + untreated (initial $n = 8$). Aim: to obtain control values for BP and survival in hypertensive animals.
2. TGR + L-NAME + untreated (initial $n = 16$). Aim: to obtain the control for BP and survival rate in untreated animals with malignant hypertension.
3. TGR + L-NAME + BAY41-8543 (initial $n = 9$). Aim: to determine BP and survival rate (indices of the course of malignant hypertension) as affected by sGC stimulation.

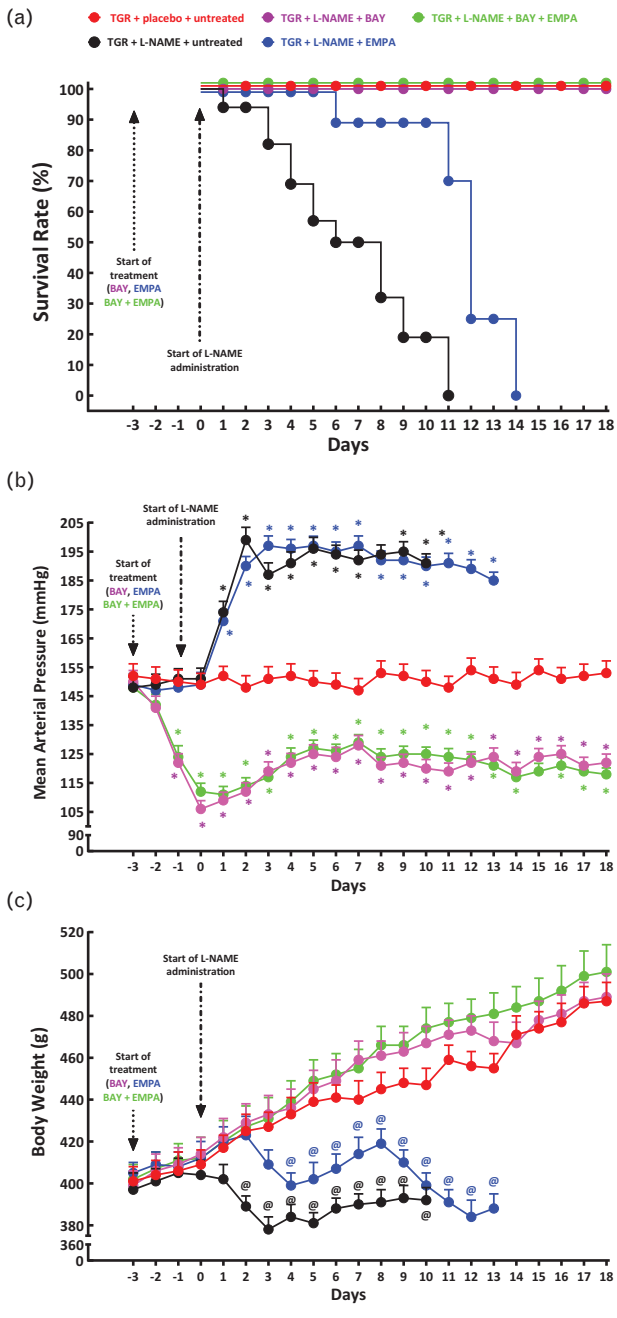

FIGURE 2 Series 1: effects of treatment on survival rate (a), mean arterial pressure (b) and body weight changes (c). BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine), a stimulator of a soluble guanylyl cyclase; EMPA, empagliflozin, a sodium-glucose cotransport type 2 inhibitor; L-NAME, Nω-nitro- L-arginine methyl ester, a nonspecific nitric oxide synthase inhibitor; TGR, heterozygous Ren-2 renin transgenic rats. $^{*}P<0.05$ compared with basal values of blood pressure (i.e. values from day −3), significance is always shown in the color appropriate to the experimental group. $^{@}P<0.05$ compared with values in control hypertensive rats (i.e. in TGR + placebo + untreated group) at the same time point.

4. TGR + L-NAME + empaglifozin (initial $n=8$). Aim: to determine BP and survival rate (indices of the course of malignant hypertension) as affected by SGLT2 inhibition.
5. TGR + L-NAME + BAY41-8543 + empaglifozin (initial $n=9$). Aim: to determine BP and survival rate (indices of the course of malignant hypertension) as affected by combined sGC stimulation and SGLT2 inhibition.

Series 2: effects of treatment with soluble guanylyl cyclase stimulator and sodium-glucose cotransporter type 2 inhibitor, alone or combined, on cardiac function and renal morphology

After defining the course of malignant hypertension-related mortality in series 1 and after detection of beneficial effects of treatment regimens on the course of survival rate in TGR treated with L-NAME, we examined mechanism(s) underlying beneficial actions of the treatment regimens and hypothesized that the actions on the malignant hypertension-related mortality might be mainly mediated by the effects on cardiac structure and function. Animals were exposed to the same experimental protocol as in series 1 (Fig. 1b) and cardiac function was explored by echocardiography on day 7 after start of L-NAME administration (at this stage, untreated TGR exposed to L-NAME administration begin markedly to die). Echocardiography was performed as described in our previous studies [59,61]. Briefly, prior to echocardiographic examination, animals were anesthetized with 4% isoflurane (IsoVet ^{R}, Piramal Healtcare, UK). During the image acquisition, rats were maintained under isoflurane anesthesia (1.5–2% Combi-vet ^{R} system, Rethacher Medical GmbH, Heitenried, Switzerland). B-mode and M-mode images were recorded in parasternal long and short-axis view and used for measurements of dimensions of left ventricle (LV) and right ventricle (RV) and parameters for evaluation of the function of the LV and RV [61]. Echocardiographic examination was done by Vevo ^{R} 2100 Imaging System with the MS250S transducer (13–24 MHz) and evaluated in VevoLab (v3.2.0., FUJIFILM VisualSonics, Inc., Toronto, Ontario, Canada). At the end, animals were killed, organ weights were again assessed, and kidneys were examined for the degree of glomerulosclerosis index (GSI) and tubulointerstitial injury (TSI) by the following methods.

The kidneys were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. The sections stained with periodic acid, for Schiff reaction, were examined and evaluated in a blind-test fashion. Fifty glomeruli in each kidney were examined on a semi-quantitative scale. The evaluation was as follows: grade 0, all glomeruli normal; grade 1, sclerotic area up to 25% (minimal sclerosis); grade 2, sclerotic area 25–50% (moderate sclerosis); grade 3, sclerotic area 50–75% (moderate-to-severe sclerosis); grade 4, sclerotic area 75–100% (severe sclerosis). The GSI was calculated using the following formula: $GSI=[(1\times n_1)+(2\times n_2)+(3\times n_3)+(4\times n_4)]/(n_0+n_1+n_2+n_3+n_4)$, where n_x is the number of glomeruli in each grade of glomerulosclerosis. Kidney cortical TSI was evaluated as defined by Nakano *et al.* [62], to determine inflammatory cell infiltration, tubular dilatation, atrophy, or interstitial fibrosis. The injury was graded semi-quantitatively using the following scale of lesions: grade 0, no abnormal findings; 1, mild (<25% of the cortex); 2, moderate (25–50% of the cortex); 3, severe (>50% of the cortex). The lesions were assessed in at least 30 random and nonoverlapping fields in the renal cortex. Thus, the maximum score for GSI is 4 and for the index of kidney TSI is 3. The values of GSI less than 0.5 and

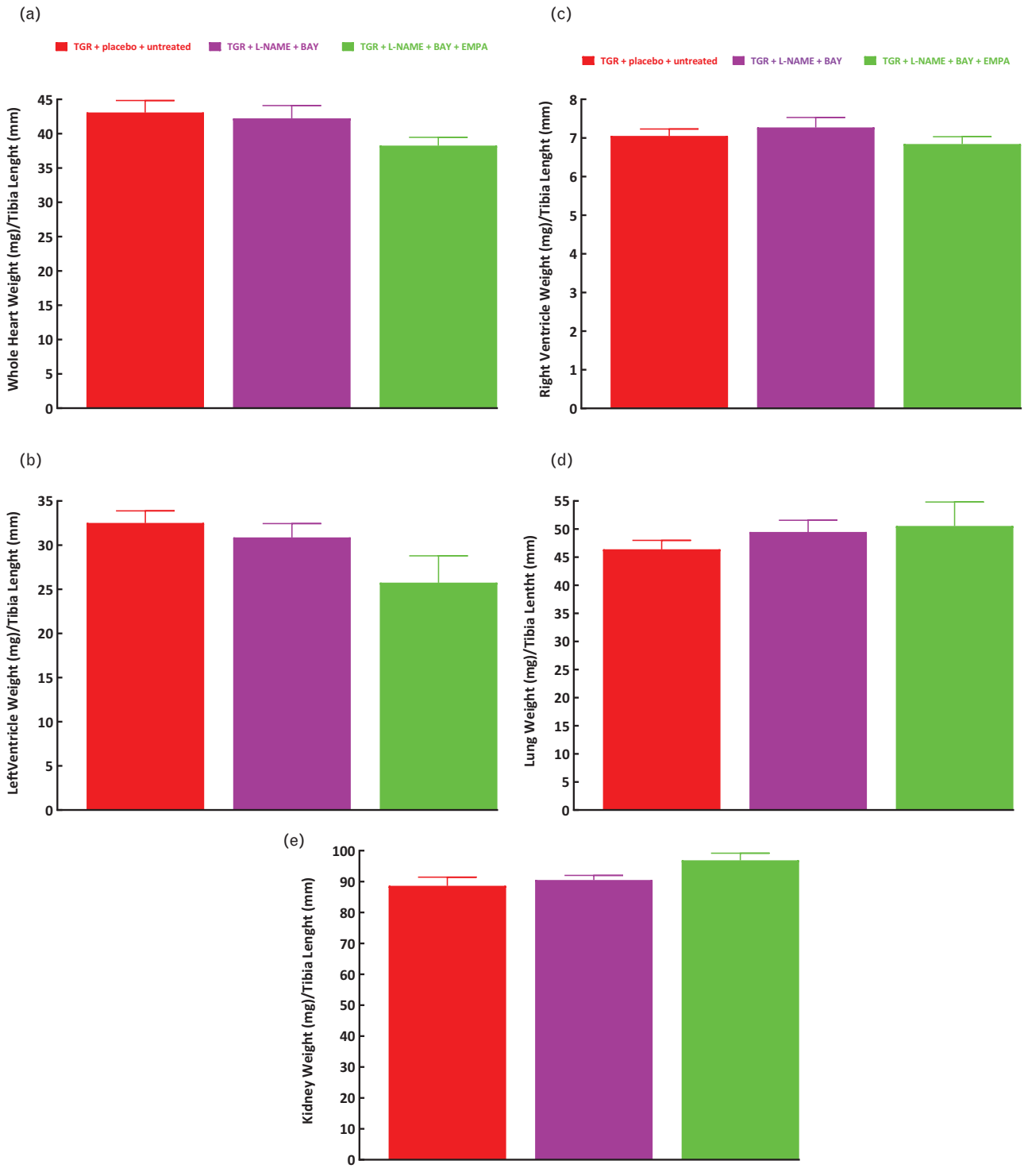

FIGURE 3 Series 1: effects of treatments on whole heart weight (a), left ventricle weight (b), right ventricle weight (c), lung weight (d) and kidney weight (e) and glomerulosclerosis index (f). BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine), a stimulator of a soluble guanylyl cyclase; EMPA, empagliflozin, a sodium-glucose cotransport type 2 inhibitor; L-NAME, Nω-nitro-L-arginine methyl ester; TGR, heterozygous Ren-2 renin transgenic rats.

TSI less than 0.4 were considered as healthy renal tissue without signs of significant renal damage. This method is always employed in our studies evaluating the degree of kidney damage [38,42,63–65].

The following experimental groups were examined:

1. TGR + placebo + untreated (initial $n = 10$). Aim: to assess cardiac function in control groups of hypertensive animals.
2. TGR + L-NAME + untreated (initial $n = 15$). Aim: to assess cardiac function in control group of untreated

animals with malignant hypertension at the onset of malignant hypertension-related mortality.

3. TGR + L-NAME + BAY41-8543 (initial $n = 12$). Aim: to assess the effects of sGC stimulation on cardiac function.
4. TGR + L-NAME + empaglifozin (initial $n = 12$). Aim: to assess the effects of SGLT2 inhibition on cardiac function.
5. TGR + L-NAME + BAY41-8543 + empaglifozin (initial $n = 12$). Aim: to evaluate the effects of combined sGC stimulation and SGLT2 inhibition on cardiac function.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism software (Graph Pad Software, San Diego, California, USA). Comparison of survival curves was performed by log-rank (Mantel–Cox) test followed by Gehan–Breslow–Wilcoxon test. Statistical comparison of other results was made by one-way ANOVA. The values are expressed as the means ± SEM and n represents the number of animals. A P value less than 0.05 was considered statistically significant.

RESULTS

Series 1: effects of soluble guanylyl cyclase stimulator and sodium-glucose cotransporter type 2 inhibitor, given alone or combined, on blood pressure and survival rate

As shown in Fig. 2a, all control hypertensive rats (i.e. TGR + placebo + untreated group) survived until the end of the study. When blocking NO signaling by adding L-NAME to the model, untreated TGR + L-NAME rats began to die rapidly, starting at day +3 and at day +11 all the animals were dead. Empagliflozin alone in the short term improved the survival rate in TGR + L-NAME animals, the first death was observed on day +6 but could not further attenuate mortality and on the day +14, all animals were dead. BAY41-8543, alone or combined with empagliflozin, abolished the mortality, and all animals survived until end of the study.

As shown in Fig. 2b, TGR + placebo-untreated animals were markedly hypertensive throughout the study (MAP ~150 mmHg) without significant alterations during the study. Administration of L-NAME in untreated rats (i.e. TGR + L-NAME + untreated group) resulted in malignant hypertension, MAP increased from basal (i.e. before L-NAME administration, on day 0) 151 ± 3–199 ± 4 mmHg on day +3 and remained hypertensive until the end of examination, even though the animals with the highest BP in this group died continuously. This was associated with a marked loss of body weight as shown in Fig. 2c; for example, on day +3, the untreated group receiving L-NAME exhibited body weight ~50 g lower than in control hypertensive rats. Empagliflozin alone did not attenuate BP increases or body weight decreases in TGR + L-NAME rats when compared with untreated TGR + L-NAME group (Fig. 2b and c). In contrast, the treatment with BAY41-8543, alone or combined with empagliflozin, resulted in TGR without L-NAME administration in profound BP decreases: MAP decreased within 3 days from basal 148 ± 3 and 151 ± 4 mmHg, respectively (i.e. values on day −3) to 106 ± 3 and 112 ± 3 mmHg, respectively, and after L-NAME administration, both treatment regimens prevented BP increases, and both groups remained clearly normotensive (Fig. 2b). In addition, treatment with BAY41-8543, alone or combined with empagliflozin, abolished body weight decreases after L-NAME administration (Fig. 2c).

Figure 3 presents organ weights at the end of this series (i.e. in animals that survived until the end of experiment). There were no significant differences in whole heart weight, left ventricle (with septum) weight, right ventricle weight, lung weight and kidney weight between experimental groups at this time point (Fig. 3a–e).

Figure 4 presents plasma and kidney ANG II at the end of this series; there were no significant differences between experimental groups.

Series 2: effects of soluble guanylyl cyclase stimulator and sodium-glucose cotransporter type 2 inhibitor, alone or combined, on cardiac function and renal morphology

As shown in Fig. 5a, on the day +7 untreated TGR + L-NAME displayed 58% survival rate. TGR + L-NAME animals

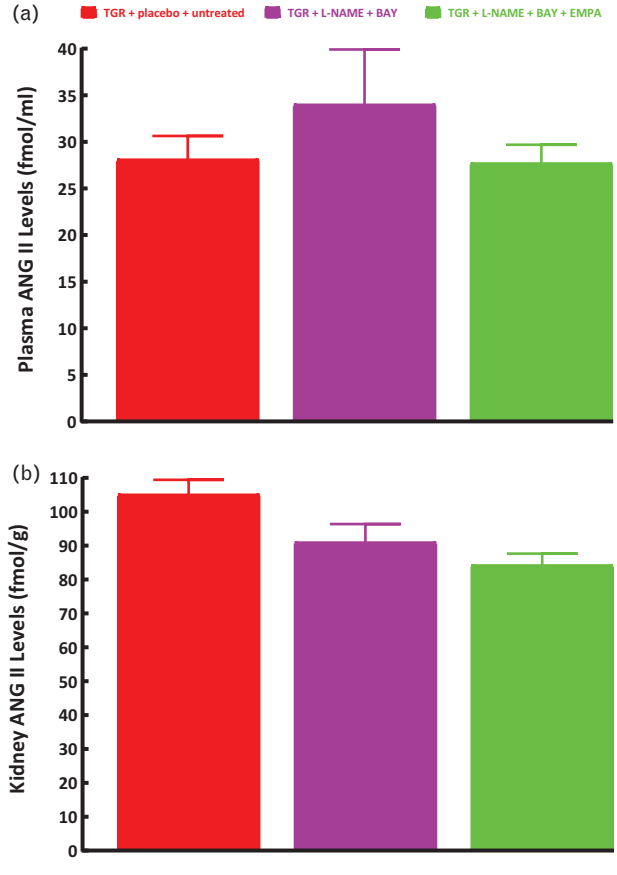

FIGURE 4 Series 1: the effects of treatment on plasma (a) and kidney (b) angiotensin II concentrations. BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine), a stimulator of a soluble guanylyl cyclase; EMPA, empagliflozin, a sodium-glucose cotransport type 2 inhibitor; L-NAME, Nω-nitro-L-arginine methyl ester; TGR, heterozygous Ren-2 renin transgenic rats.

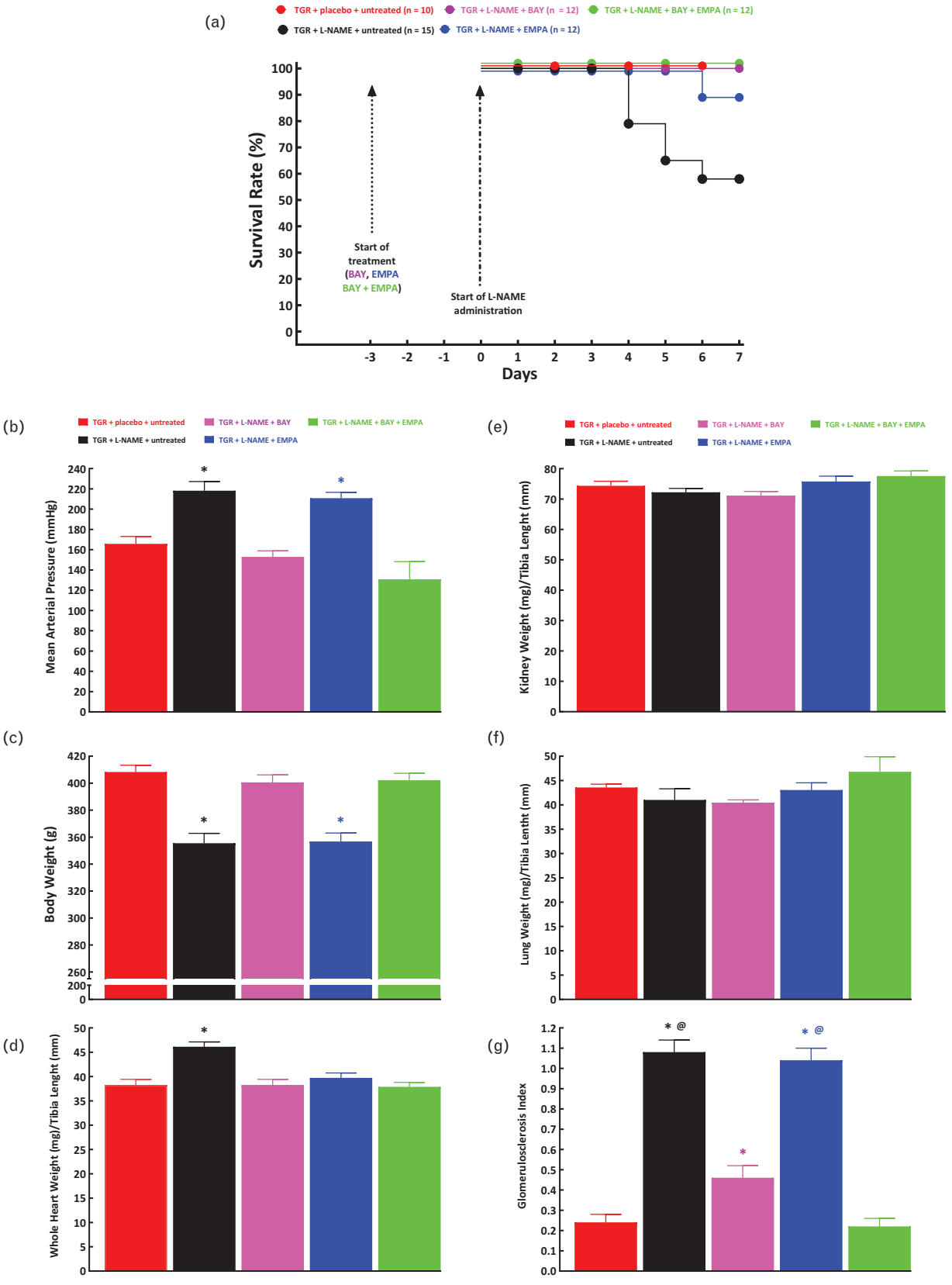

FIGURE 5 Series 2: the effects of treatment on survival rate (a), mean arterial pressure (b), body weight (c), whole heart weight (d), kidney weight (e), lung weight (f) and glomerulosclerosis index (g). BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine), a stimulator of a soluble guanylyl cyclase; EMPA, empagliflozin, a sodium-glucose cotransport type 2 inhibitor. L-NAME, Nω-nitro- L-arginine methyl; TGR, heterozygous Ren-2 renin transgenic rats. $^{*}P < 0.05$ compared with values in control hypertensive rats (i.e. TGR + placebo + untreated group). Significance is shown in the color appropriate to the experimental group.

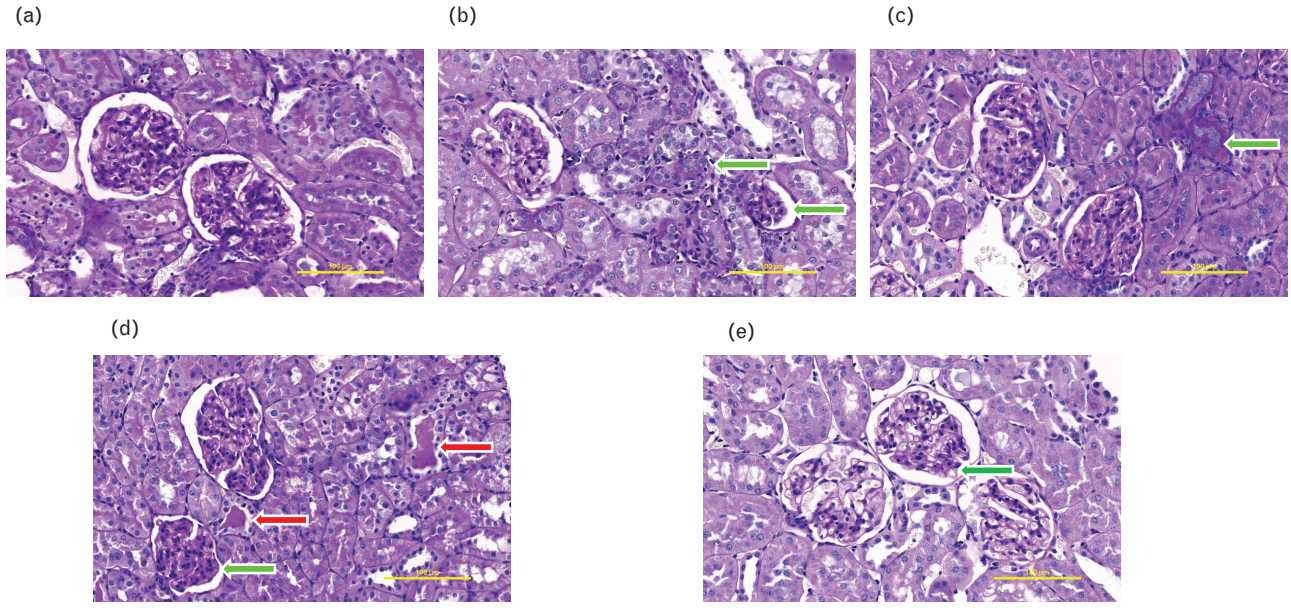

FIGURE 6 Series 2: Representative histological images of the renal cortex in (a) control hypertensive rats, that is, heterozygous Ren-2 renin transgenic rats (TGR) + placebo + untreated – with normal renal morphology. (b) TGR + administration of Nω-nitro-L-arginine methyl ester (L-NAME) without treatment (i.e. TGR + L-NAME + untreated) – show substantial glomerular damage with signs of focal segmental glomerular sclerosis changes accompanied by small areas of tubular atrophy and tubulointerstitial changes (fibrosis), green arrows highlight the most prominent changes in this experimental group. (c) TGR + L-NAME + treated with BAY41-8543-(2-[1-[2-flurophenyl) methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine) (BAY) (i.e. TGR + L-NAME + BAY) – show principally same changes as observed in TGR + L-NAME + untreated group, and green arrows again highlights the typical changes for this group. (d) TGR + L-NAME + treated with empagliflozin, a sodium-glucose cotransport type 2 inhibitor (EMPA) (i.e. TGR + L-NAME + EMPA), principally the same changes as observed in TGR + L-NAME + untreated group, green arrow shows the typical change and red arrows show protein casts. (e) TGR + L-NAME + BAY + EMPA, principally the same findings as observed n TGR + L-NAME + untreated group, green arrow shows the typical change. Bar scale 100 μm (shown in yellow).

treated with empagliflozin exhibited 89% survival rate, and the TGR + L-NAME treated with BAY41-8543, alone or combined with empagliflozin, displayed 100% survival rate, confirming that untreated TGR + L-NAME rats exhibited an onset of decompensation of malignant hypertension, whereas the treated TGR + L-NAME group was still in the compensation phase.

As shown in Fig. 5b, untreated TGR + L-NAME rats exhibited markedly elevated MAP as compared with control hypertensive rats (218 ± 9 vs. 166 ± 7 mmHg, $P < 0.05$). In TGR + L-NAME rats, empagliflozin alone did not alter MAP as compared with untreated TGR + L-NAME group. TGR + L-NAME rats treated with BAY41-8543, alone or combined with empagliflozin showed markedly lower MAP as compared with untreated TGR + L-NAME group; there was a clear tendency for lower MAP as compared with control hypertensive rats, but at this time point, the difference did not reach significance level.

As shown in Fig. 5c, untreated TGR + L-NAME and TGR + L-NAME rats treated with empagliflozin displayed lower body weight as compared with control hypertensive rats.

As shown in Fig. 5d, untreated TGR + L-NAME animals displayed markedly higher whole heart weight as compared with all the other groups.

There were no significant differences in kidney weight and lung weight between experimental groups (Fig. 5e and f).

As shown in Fig. 5g, untreated TGR + L-NAME rats displayed severe renal damage as compared with control hypertensive rats: GSI was about four-fold higher and clearly in the range of significant renal glomerular injury. In TGR + L-NAME rats, no treatment regimen altered GSI as compared with untreated TGR + L-NAME group. The same was valid for TSI (data not shown). Representative images of renal parenchyma are shown in Fig. 6.

Table 1 and Fig. 7 present an evaluation of cardiac structure and function by echocardiography. As shown in Fig. 7a–c, untreated TGR + L-NAME rats displayed impairment of LV systolic function as compared with control hypertensive rats: this was documented by decreased LV fractional shortening, LV ejection fraction and LV stroke volume. As expected, impairment of LV systolic function in untreated TGR + L-NAME rats resulted in decreased cardiac output as compared with control hypertensive rats. In TGR + L-NAME rats, empagliflozin alone did not prevent a decrease in LV systolic function and cardiac output. In contrast, in TGR + L-NAME rats, BAY41-8543 alone or combined with empagliflozin prevented decreases in LV systolic function and cardiac output (Fig. 7a–d). As shown in Fig. 7e, acceleration of cardiac hypertrophy in untreated TGR + L-NAME group (as documented in Fig. 5c) was accompanied by a significant rise in the relative LV wall thickness, indicating further acceleration of LV concentric chamber remodeling. Empagliflozin alone in TGR + L-NAME did not influence this process, but BAY41-8543 alone or combined with empagliflozin in TGR + L-NAME abolished the increases in relative LV wall thickness (Fig. 7e).

DISCUSSION

The main finding of our present study is that the treatment with the sGC stimulator BAY41-8543, which shares the

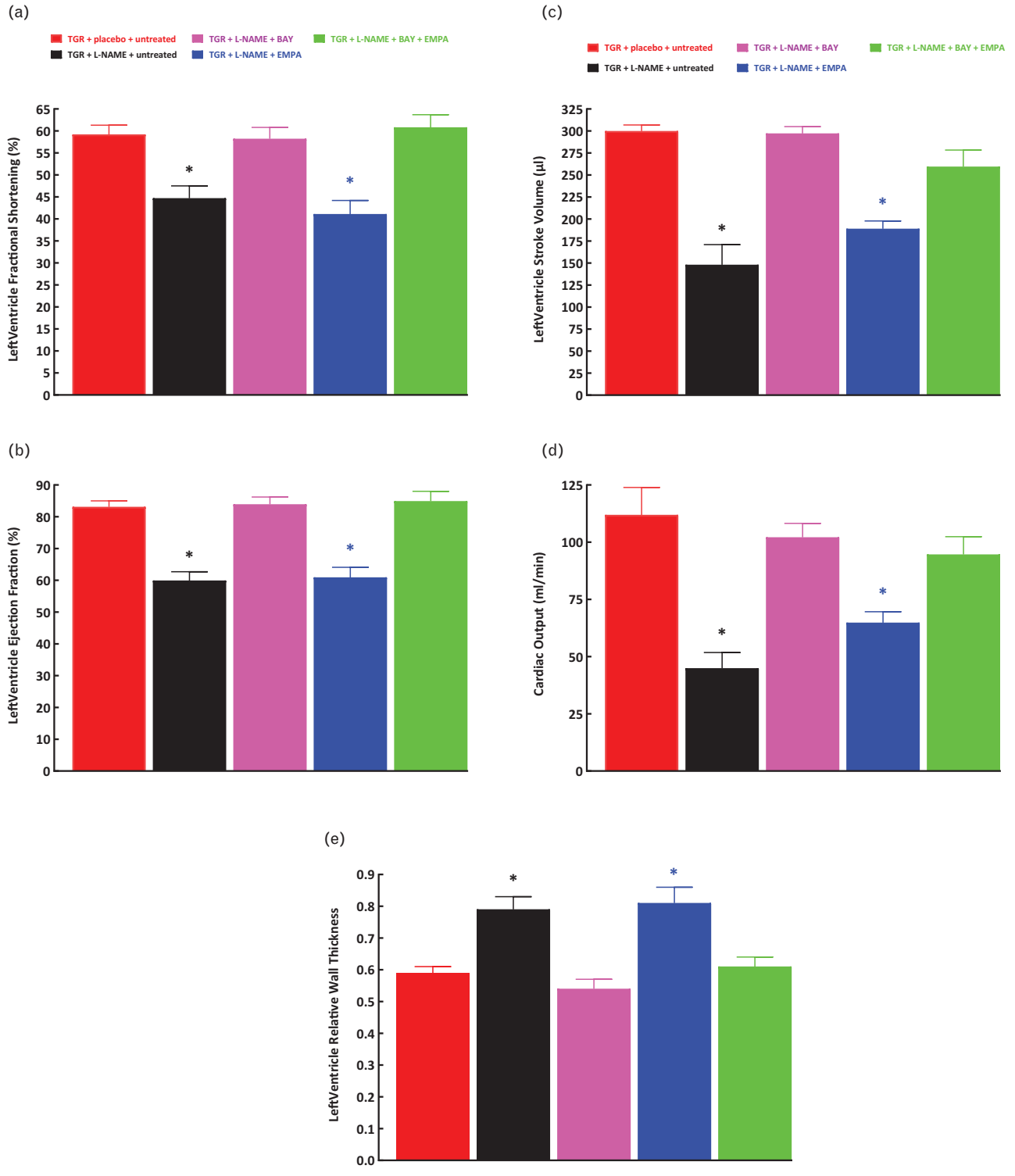

FIGURE 7 Series 2: the effects of treatment on the fractional shortening (a), ejection fraction (b), stroke volume (c), cardiac output (d) and relative wall thickness (e) of the left ventricle. BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine), a stimulator of a soluble guanylyl cyclase; EMPA, empagliflozin, a sodium-glucose cotransport type 2 inhibitor; L-NAME, Nω-nitro-L-arginine methyl ester; TGR, heterozygous Ren-2 renin transgenic rats. *$P < 0.05$ compared with values in control hypertensive rats (i.e. TGR + placebo + untreated group). Significance is shown in color appropriate to the experimental group.

same mode of action as the sGC stimulator vericiguat [25], either alone or combined with SGLT2 inhibitor empagliflozin completely prevented malignant hypertension-related mortality. In addition, the prevention of the onset of the mortality, these two treatment regimens also prevented blood pressure increases after L-NAME administration in TGR and even decreased MAP. The treatments also abolished impairment of cardiac LV function, evidently preventive actions on malignant hypertension-related morbidity.

TABLE 1. Series 2: echocardiographic analysis performed 10 days after initiation of appropriate treatment regimens and 7 days after start of L-NAME administration

	Group				
	TGR + placebo + untreated	TGR + L-NAME + untreated	TGR + L-NAME + BAY	TGR + L-NAME + EMPA	TGR + L-NAME + BAY + EMPA
Heart rate (/s)	369 ± 10.1	344 ± 11.2	345 ± 6.7	347 ± 10.1	355 ± 5.4
Left ventricle diastolic diameter (mm)	8.08 ± 0.13	6.99 ± 0.1***	8.09 ± 0.09	7.14 ± 0.1***	7.55 ± 0.18*
Left ventricle systolic diameter (mm)	3.54 ± 0.13	4.3 ± 0.32*	3.63 ± 0.12	4.12 ± 0.21	3.33 ± 0.11
Left anterior wall thickness in diastole (mm)	2.04 ± 0.06	2.12 ± 0.04	1.88 ± 0.05	2.05 ± 0.05	1.92 ± 0.07
Left anterior wall thickness in systole (mm)	3.76 ± 0.09	3.37 ± 0.17**	3.49 ± 0.07	3.28 ± 0.06***	3.4 ± 0.06**
Left posterior wall thickness in diastole (mm)	2.37 ± 0.06	2.67 ± 0.08	2.21 ± 0.11	2.61 ± 0.08	2.38 ± 0.09
Left posterior wall thickness in systole (mm)	4.08 ± 0.06	3.68 ± 0.08*	3.79 ± 0.1	3.79 ± 0.16	3.98 ± 0.06
Right ventricle basal diameter in diastole (mm)	3.79 ± 0.11	3.51 ± 0.23	4.01 ± 0.13	3.56 ± 0.12	4.24 ± 0.09
Right ventricle mid diameter in diastole (mm)	3.58 ± 0.09	2.91 ± 0.13***	3.71 ± 0.09	2.9 ± 0.12***	3.91 ± 0.07
Right ventricle longitudinal diameter in diastole (mm)	11.9 ± 0.23	11.1 ± 0.14*	11.9 ± 0.07	11.8 ± 0.17	11.8 ± 0.21
Right ventricle diastolic area (mm^2)	41.5 ± 1.37	33.9 ± 2.22*	43.5 ± 1.17	36.9 ± 1.92	47.2 ± 1.43
Right ventricle fractional area change (%)	58.4 ± 1.88	36.4 ± 3.61***	53.1 ± 1.61	44.7 ± 1.58***	49.2 ± 1.55**

The values are the means ± SEM. BAY, BAY41-8543-(2-[1-[2-flurophenyl)methyl]-1H-pyrazol][3,4b]pyridine-3-yl]-5(4-morpholinyl)-4,6-pyrimidinedaiamine) a stimulator of a soluble guanylyl cyclase; EMPA, empaglifozin a sodium-glucose cotransport type 2 inhibitor; L-NAME, Nω-nitro-L-arginine methyl ester a nonspecific nitric oxide synthase inhibitor; TGR. heterozygous Ren-2 renin transgenic rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with values in control hypertensive rats (i.e. TGR + placebo + untreated group).

Although empagliflozin is the SGLT2 inhibitor with one of the best outcomes with regard to treatment of the cardiorenal system [43–48,66], the treatment with empagliflozin alone did not show sustained protective action on the malignant hypertension-related mortality and organ damage. These results do, therefore, not support second hypothesis, namely that the SGLT2 inhibitor will augment the beneficial actions of sGC stimulation on the course of malignant hypertension-related mortality.

However, one limitation of the current animal study might be, the mechanism(s) driving the disease severity in this model and underlying the beneficial effects of sGC stimulation.

Most probably, disease severity and the majority of beneficial effects were related to the antihypertensive actions of BAY41-8543: it will be noticed that in untreated TGR receiving L-NAME, MAP was about 60 mmHg higher than in NO-deficient TGR treated with BAY41-8543 given alone or combined with empagliflozin. In addition, the animals treated with sGC stimulator were clearly rescued from developing hypertension. In contrast, BAY 41-8543-treated animals were normotensive, with BP about 30 mmHg lower than observed in control hypertensive rats (i.e. in TGR + placebo + untreated group). Our results, therefore, suggest that beneficial effects of sGC stimulation on the course of malignant hypertension and malignant hypertension-related organ damage are predominantly BP-dependent.

This is further supported by the marked and immediate improvement of LV systolic function and normalization of cardiac output, likely the consequence of reduced afterload related to the substantial BP decreases in groups treated with BAY 41-8543.

However, the remarkable BP-lowering effects of BAY41-8543 in TGR treated with L-NAME also suggests that under conditions when hypertension is accompanied by endothelial dysfunction and NO deficiency, which is often the case in malignant hypertension, the use of sGC stimulator is a very suitable and causal approach for the treatment of malignant hypertension, because its action is NO-independent [25]. Moreover, our present findings showing that the treatment with BAY41-8543, alone or combined with empagliflozin, did not alter ANG II concentrations indicate that sGC stimulation did not substantially alter RAS activity in this model of malignant hypertension. Therefore, antihypertensive and organ-protective actions are unlikely to be related to alterations of the RAS.

The overwhelming efficacy of BAY 41-8543 sGC stimulator treatment, which due to the unique mode of action seems tailored for treatment of malignant hypertension caused by endothelial dysfunction, made it impossible to answer the second question on additive or synergistic effects of the SGLT2 inhibitor empagliflozin.

This is definitely a limitation of the current studies, which might be addressed in the future by potential down-titration of the sGC stimulator dose, which result in submaximal efficacy response. Also, longer treatment durations with the combination might be for future consideration, but most likely, a different animal model in which disease cause rather than hypertension have to be considered.

In conclusion, our present results clearly demonstrate that treatment with the sGC stimulator BAY41-8543, resulted in marked protection against malignant hypertension, malignant hypertension-related mortality and malignant hypertension-related cardiac damage. The NO-independent mechanism of action of sGC stimulators might be a causal and precise therapy of malignant hypertension driven by endothelia dysfunction and NO decline. In general, these findings suggest that targeting the NO/sGC/cGMP pathway by employing sGC stimulator could be a promising therapeutic means for the treatment of malignant hypertension, whereas additional benefits of SGLT2i on top of sGC stimulators have to be shown yet.

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Conflicts of interest

There are no conflicts of interest.

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