Comparison of the antiremodeling effects of losartan and mirabegron in a rat model of uremic cardiomyopathy

Summary of the Ph.D. Thesis

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1. Introduction

The clinical syndrome of chronic kidney disease (CKD) is defined by abnormalities of kidney structure and/or deterioration of kidney function present for at least 3 months with implications for the individual's health. CKD is an emerging public health burden globally, affecting 1 out of 10 people due to the growing prevalence of its primary causes, including aging, diabetes mellitus, and hypertension. In CKD, the continuous and permanent deterioration of the glomerular, tubular, and endocrine functions of the kidney lead to end-stage renal disease (ESRD). At this last stage of CKD, the glomerular filtration rate (GFR) is below 15 mL/min/1.73 m^2, and kidney replacement therapy is required for the patients' survival. As the number of functional nephrons declines, several systemic complications could arise. Deterioration in vital renal functions, such as regulation of fluid and electrolyte levels, acid-base balance, endocrine functions affecting bone integrity or erythropoiesis, lipid metabolism, and blood pressure, could lead to systemic complications affecting the whole organism. Notably, CKD patients have a 5- to 10-fold higher risk of developing cardiovascular diseases (CVDs) than the age-matched non-CKD population. Indeed, CVDs are the leading cause of mortality in all stages of CKD. Moreover, CKD is recognized as an independent risk factor for CVDs due to its role in the development of left ventricular hypertrophy (LVH) and the acceleration of atherosclerosis. The CKD-associated chronic structural, functional, and electrophysiological remodeling of the heart is called uremic cardiomyopathy (i.e., type 4 cardiorenal syndrome). In the case of type 4 cardiorenal syndrome, CKD is the primary disease leading to progressive cardiovascular impairment. Uremic cardiomyopathy is characterized by diastolic dysfunction (DD), LVH, capillary rarefaction, endothelial dysfunction, and fibrosis in the stage of heart failure with preserved ejection fraction (HFpEF). Both preclinical and clinical studies showed that LVH could develop in CKD regardless of pressure- and volume-overload. Moreover, the regression of LVH was observed after kidney transplantation in ESRD patients. This observation suggests that specific factors related to CKD itself, such as the uremic toxins, contribute to the multifactorial pathogenesis of LVH. The prevalence of LVH significantly increases with the progression of CKD. Indeed, LVH is present in 50–70% of CKD patients and up to 90% in dialyzed patients with ESRD. With the progression of CKD, cardiac fibrosis becomes more prominent, leading to systolic dysfunction in the phase of heart failure with reduced ejection fraction (HFrEF).

Currently, there are no specific targeted therapeutic strategies available to prevent or treat uremic cardiomyopathy. The current cornerstone treatment options of uremic cardiomyopathy

include drugs applied in heart failure with different etiologies and pharmaceuticals targeting common risk factors of CKD and CVDs (e.g., hypertension, diabetes mellitus, or hyperlipidemia). However, these standard therapeutic options mean only symptomatic treatments slowing down the progression of CKD. Multiple factors might contribute to the development of uremic cardiomyopathy; however, the precise molecular mechanisms are not entirely clear yet. These factors include non-CKD-specific mechanisms such as hemodynamic overload with over-activation of the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system, hypertension, endothelial dysfunction, inflammation, and increased nitro-oxidative stress and CKD-specific factors such as circulating uremic toxins and renal anemia. In uremic cardiomyopathy, the chronically elevated angiotensin-II levels might stimulate several pathological mechanisms such as inflammation, nitro-oxidative stress, decreased nitric oxide (NO) bioavailability via angiotensin-II receptor type 1 (AT1) receptors, ultimately leading to fibrosis in the heart and kidneys. Therefore, RAAS inhibitors, including angiotensin-II receptor blockers (ARBs), are widely used to slow down the progression of heart failure or CKD in clinical practice. Indeed, losartan, a well-known and widely applied antihypertensive ARB improved LVH and myocardial fibrosis in ESRD patients with hypertension. Despite the broad availability of standard heart failure medications such as ARBs, cardiovascular morbidity and mortality among CKD patients remained high. Therefore, the administration of novel agents that ameliorate or prevent the progression of uremic cardiomyopathy is urgently needed.

In the treatment of HFpEF, enhancing NO bioavailability is considered a promising novel approach. Mirabegron is a β3-adrenergic receptor (β3-AR) agonist recently used in the clinical treatment of overactive bladder syndrome, improving the bladder filling capacity by relaxing the detrusor muscle via β3-AR. In healthy heart tissue, the expression of β3-AR is considered low in atrial and ventricular myocytes but more abundant in non-cardiomyocytes, including endothelial cells. In contrast to β1- and β2-ARs, cardiac β3-AR abundance increases as a counterregulatory mechanism to prevent adrenergic overactivation in chronic ischemia and heart failure. In preclinical models, the β3-AR agonists attenuated cardiac hypertrophy and fibrosis, and improved cardiac contractility via coupling of β3-AR to the endothelial nitric oxide synthase (eNOS)/cGMP pathway and activating Na^+/K^+-ATPase-mediated Na^+ export in the cardiomyocytes. Moreover, the antioxidant effects of the β3-AR signaling may protect the heart from elevated nitro-oxidative stress and the consecutive pro-inflammatory processes. It was demonstrated that angiotensin-II administration did not induce cardiac fibrosis and hypertrophy in mice with cardiomyocyte-specific expression of human β3-AR. It has also

been described in pancreatic and lung tissues of male apoE knock-out mice that chronic administration of the β3-AR agonist BRL37344 could down-regulate the AT1 receptors. These results suggest that chronic β3-adrenoceptor activation can regulate the expression of angiotensin-II receptors, and these interactions may play a protective role in the pancreas and lungs or other tissues such as the heart. Based on these experimental data, the promising therapeutic effects of the β3-AR agonist mirabegron are investigated in human clinical trials on the development of LVH, HFpEF, and HFrEF. Notably, CKD patients are excluded from these clinical trials. Thus, the effects of mirabegron were not investigated in a selected patient population with mild to moderate uremic cardiomyopathy or in experimental CKD. Therefore, we aimed to compare the antiremodeling effects of the ARB losartan used in standard heart failure therapy and the novel β3-AR agonist mirabegron in a rat model of uremic cardiomyopathy.

2. Aims of the thesis

Our present thesis aimed to compare the effects of losartan and mirabegron on routine serum and urine laboratory parameters, cardiac morphology and function, as well as left ventricular expression changes of selected genes and proteins associated with diastolic function, cardiac hypertrophy, fibrosis, inflammation, and nitro-oxidative stress in a rat model of uremic cardiomyopathy.

3. Materials and methods

This investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and was approved by the Animal Research Ethics Committee of Csongrád County (XV./799/2019) and the University of Szeged in Hungary. All institutional and national guidelines for the care and use of laboratory animals were followed. The authors complied with the ARRIVE guidelines.

3.1. 5/6th nephrectomy: A total of 45 adult male Wistar rats (*Rattus norvegicus*, 8 weeks old, 280-340 g) were enrolled in this study. Experimental CKD was induced by 5/6th nephrectomy in two phases after pentobarbital anesthesia. First, the 1/3 left kidney on both ends was excised, and one week later, the right kidney was removed. During sham operations, only the renal capsules were removed. After both surgeries, povidone-iodine was applied on the skin's surface, and as a post-operative medication nalbuphine hydrochloride analgesics and enrofloxacin antibiotics were administered for 4 days.

3.2. Follow up and pharmacological treatment of the animals: On the first day of the 5th follow-up week, rats were divided into the following groups and treated via oral gavage daily for 8 weeks: 1) sham-operated group treated with *per os* tap water (2 mL/kg/day, *n*=10), 2) CKD group treated with *per os* tap water (2 mL/kg/day, *n*=13), 3) CKD group treated with *per os* losartan (10 mg/kg/day dissolved in tap water, 2 mL/kg end volume, *n*=12), and 4) CKD group treated with *per os* mirabegron (10 mg/kg/day in tap water, 2 mL/kg end volume, *n*=10). In our study, 6 animals died from the nephrectomized groups (2 animals in the CKD group, 1 animal in the losartan-treated CKD group, and 3 animals in the mirabegron-treated CKD group).

3.3. Transthoracic echocardiography: At weeks 4 and 12, cardiac morphology and function were assessed by transthoracic echocardiography. Rats were anesthetized with 2% isoflurane and two-dimensional, M-mode, Doppler, tissue Doppler, and 4 chamber-view images were performed by the criteria of the American Society of Echocardiography with a Vivid IQ ultrasound system using a phased array 5.0–11 MHz transducer. Data of three consecutive heart cycles were analyzed by an experienced investigator in a blinded manner.

3.4. Serum and urine laboratory parameters: Blood was collected from the saphenous vein at week 4 and from the abdominal aorta at week 13 to measure serum parameters. The animals were placed into metabolic cages for 24 h at weeks 4 and 12 to measure urine creatinine and protein levels. Serum carbamide and creatinine levels were quantified by a kinetic UV method. Serum sodium, potassium, and chloride levels were determined by indirect potentiometry using ion-selective electrodes. The serum calcium, magnesium, phosphate levels were quantified by complex formation methods. Urine creatinine and urine protein levels were measured by standard laboratory methods. Serum total cholesterol, HDL-cholesterol, and triglyceride levels were measured by enzymatic colorimetric assays. LDL-cholesterol was calculated according to the Friedewald formula. Total blood count and hematocrit were measured from whole blood by a hematology analyzer.

3.5. Blood pressure measurement: At week 13, invasive blood pressure measurements were performed in a subgroup of animals by inserting a PE50 polyethylene catheter into the left femoral artery under pentobarbital anesthesia.

3.6. Tissue harvesting: At week 13, hearts were isolated, then left ventricular (LV) samples were fixed in 4% buffered formalin for histology or freshly frozen in liquid nitrogen until further biochemical measurements.

3.7. Hematoxylin-eosin (HE) and picrosirius red and fast green (PSFG) staining: The development of LVH in CKD was verified by the measurement of cardiomyocyte diameters and cross-sectional areas on HE stained slides using the Biology Image Analysis Software (BIAS) by Single-Cell Technologies Ltd. Cardiac fibrosis was assessed on PSFG slides with an in-house developed program.

3.8. mRNA expression profiling by qRT-PCR: Total RNA was isolated from the left ventricles and reverse transcribed, then the myocardial expression of the β3-adrenergic receptor (*Adrb3*) and selected genes associated with the RAAS [i.e., angiotensinogen (*Agt*) angiotensin-II receptor type 1a (*Agtr1a*)], left ventricular hypertrophy [i.e., α- and β myosin heavy chains (*Myh6* and *Myh7*)], tissue inflammation [i.e., interleukin-1 and -6 (*IL1* and *IL6*) and tumor necrosis factor-alpha (*Tnf-α*)] and fibrosis [i.e., collagen type 1 alpha 1 chain (*Col1a1*) and connective tissue growth factor (*Ctgf*)], heart failure [i.e., A- and B types natriuretic peptides (*Nppa* and *Nppb*)], and nitro-oxidative stress [i.e., inducible nitric oxide synthase (*Nos2*) and NADPH oxidase type 4 (*Nox4*)]. Peptidyl-prolyl isomerase A (*Ppia*) was used as a housekeeping control gene for normalization.

3.9. Western blot: Standard Western blot technique was used in the case of β3-AR with actin, eNOS, phospho-eNOS, sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) with α-tubulin, and phospholamban (PLN), phospho-PLN with GAPDH loading background to measure LV protein expressions. Left ventricular samples were homogenized, and after quantifying the supernatants' protein concentrations, sodium dodecyl-sulfate polyacrylamide gel electrophoresis was performed, followed by the transfer of proteins onto a nitrocellulose membrane. Membranes were blocked and then incubated with primary antibodies overnight. Then the membranes were incubated with secondary antibodies, and the fluorescent signals were detected by the Odyssey CLx machine.

3.10. Statistical analysis: $p<0.05$ was accepted as a statistically significant difference. One-Way ANOVA was used to determine the statistical significance between all measured parameters within each time point. Two-way repeated-measures ANOVA was used to determine the effects of CKD and the treatments on serum, urine, and echocardiographic parameters between week 4 and endpoint follow-up data. Holm-Sidak test was used as a *post hoc* test.

4. Results

4.1. Characteristic laboratory, echocardiographic, mRNA, and protein expression changes in uremic cardiomyopathy

In the 5/6th nephrectomized rats, uremic cardiomyopathy developed with similar laboratory and cardiac alterations to those in CKD-induced HFpEF patients. Our present findings on the characteristic laboratory and echocardiographic parameters in CKD are consistent with the literature data and our previous results in 5/6th nephrectomized rats. In this study, week 4 laboratory parameters confirmed that 5/6th nephrectomized animals developed renal failure at the same severity in each CKD group before starting the drug administrations. We have found here characteristic laboratory changes in CKD, including increased serum urea and creatinine concentrations, increased urine protein levels and decreased creatinine clearance 13 weeks after the operations. Moreover, renal anemia, hypercalcemia, and hypercholesterolemia developed in the 5/6th nephrectomized animals. In our CKD model, echocardiography confirmed the presence of uremic cardiomyopathy characterized by LVH and DD. Histology confirmed LVH at the cellular level and revealed interstitial fibrosis. According to these findings, the severity of CKD in our model corresponds to the human G2 or G3a CKD stages with mildly or moderately decreased kidney function. Notably, severe hypertension is usually not a feature of the 5/6th nephrectomy-induced CKD models. Indeed, our CKD model showed only a tendency of increase in blood pressure values. Therefore, DD might be developed due to LVH and fibrosis without severe hypertension. Both preclinical and clinical studies showed that CKD-specific and non-specific risk factors such as uremic toxins, renal anemia, the over-activation of the RAAS and sympathetic nervous system, elevated nitro-oxidative stress, decreased NO levels, increased systemic and tissue inflammation could provoke the development of uremic cardiomyopathy. Indeed, our CKD model showed significantly elevated white blood cell count and overexpressed pro-inflammatory cytokines, including *IL1*, *IL6*, and *Tnf-α* in the left ventricle, indicating systemic and tissue inflammation. The interplay of increased nitro-oxidative stress and inflammation can be an essential contributor to LVH in CKD and was linked to the progression of HFpEF. NADPH oxidases (NOX), producing superoxide anion, may also be involved in the development of cardiac hypertrophy, interstitial fibrosis, or contractile dysfunction. Accordingly, LV *Nox4* expression showed a statistically non-significant increase in our CKD model. Reduced NO bioavailability due to increased superoxide levels and consequential peroxynitrite formation might contribute to cardiomyocyte stiffness and fibrosis, leading to diastolic dysfunction and microvascular

endothelial dysfunction in HFpEF and uremic cardiomyopathy. Our CKD model showed a decreased myocardial eNOS protein level, probably, due to the capillary rarefaction and hypertrophy in CKD, and a significantly elevated eNOS phosphorylation on the main activation site (Ser1177). The latter could be a compensatory mechanism to increase NO production in uremic cardiomyopathy. Accordingly, the phospho-eNOS/eNOS ratio was tendentiously increased in CKD as compared to the sham operated-group. Our results on eNOS expression and activity in uremic cardiomyopathy are in line with the literature data. Furthermore, eNOS uncoupling leads to ROS/RNS production instead of NO, increasing the nitro-oxidative stress and hypertrophic remodeling. According to studies using animal models and human myocardial samples, the up-regulation of iNOS (i.e., *Nos2*) can contribute to the dysregulation of NO production in oxidative and pro-inflammatory states leading to increased peroxynitrite production, which can be associated with pathologic cardiac remodeling. Indeed, in our present study, *Nos2* expression was significantly increased in uremic cardiomyopathy. On the other hand, eNOS-derived NO production was demonstrated to enhance LV relaxation via PLN-mediated SERCA Ca^{2+} reuptake. Decreased SERCA2a expression and disturbed calcium homeostasis could also be associated with impaired relaxation and declining systolic function in later stages of HF. In contrast, a study found that SERCA2a and its regulator, PLN did not decrease in early isolated DD induced by aortic stenosis. Others demonstrated in $5/6^{th}$-nephrectomy-induced CKD in rats that reduced phosphorylation of PLN could lead to reduced SERCA activity 8 weeks after the operation. However, they found increased PLN phosphorylation 24 weeks after the operation, pointing out the time-dependency of PLN phosphorylation and SERCA activity in uremic cardiomyopathy. In our study, there was no significant change in SERCA2a and PLN expressions 13 weeks after the operations in CKD. Losartan and mirabegron did not change the SERCA expression and phospho-PLN/PLN expressions in CKD, suggesting that these proteins did not play a crucial role in the effects causing improved diastolic function in the losartan and mirabegron-treated groups.

4.2. Effects of chronic ARB losartan administration in CKD

Most of the standard therapies in uremic cardiomyopathy are supportive and used to modulate comorbidities or heart failure slowing down the progression of the condition. RAAS inhibitors, including ARBs, are cornerstone therapies to reduce proteinuria, CKD progression, and cardiovascular risk. The ARB losartan was reported to ameliorate LVH and myocardial fibrosis development and improve cardiac function in $5/6^{th}$ nephrectomized rats and ESRD patients. In our present study, losartan failed to improve the main routine laboratory

parameters of kidney function, including creatinine clearance, serum carbamide and creatinine levels. However, it reduced the urine volume and the remnant kidney weight compared to the CKD group showing a mild renoprotective effect. Evidence suggests that chronic over-activation of the RAAS and the sympathetic nervous system in heart failure also stimulates the inflammatory processes and increases nitro-oxidative stress, further aggravating each other. Angiotensin-II has been reported to activate cardiac NADPH-oxidase via AT1 receptor and, subsequently, the over-production of ROS/RNS. The increased nitro-oxidative stress could trigger the production of pro-inflammatory mediators, such as IL1, IL6, TNF-α, and tissue growth factor-beta (TGF-β), and the inflammatory cytokines could decrease eNOS expression and NO bioavailability, contributing to cardiac remodeling and HF. Therefore, AT1 blockade by losartan is a rational therapeutic option to ameliorate cardiac remodeling by reducing nitro-oxidative stress, inflammatory processes and improve NO bioavailability in uremic cardiomyopathy. Indeed, in our present study, losartan improved the DD, LVH, and cardiac fibrosis, probably via blocking the AT1 receptor-mediated nitro-oxidative (*Nox4* and *Nos2*) and inflammatory (*IL1, IL6,* and *Tnf-α*), and eNOS-associated mechanisms in our CKD model. Interestingly, it has been reported that another ARB, valsartan combined with the neprilysin inhibitor sacubitril (LCZ696), attenuated cardiac hypertrophy and fibrosis in 5/6th nephrectomized rats. Human phase I clinical trials are currently observing the effect of sacubitril/valsartan on advanced CKD patients with HF (NYHA class II-IV) (trial No.: NCT03771729 and NCT04218435). Taken together, our present results are in line with the literature data on the antiremodeling effects of ARBs in uremic cardiomyopathy.

4.3. Effects of chronic β3-AR agonist mirabegron therapy in a rat model of uremic cardiomyopathy

Modulation of NO signaling is considered one of the novel promising approaches in HFpEF treatment. β3-AR agonists are reported to ameliorate cardiac remodeling, diastolic and systolic dysfunction via the β3-AR receptor coupled eNOS-derived NO production beyond other mechanisms such as preserving the function of the Na^+/K^+-ATP-ase, antioxidant effects via the AKT-NO-mPTP signaling, and influencing the fatty acid metabolism. Currently, phase II and III clinical trials are investigating the effect of mirabegron on the development of LVH and HFpEF (NYHA class I-II) (Beta3_LVH trial, trial No.: NCT02599480), and in patients with HFrEF (NYHA class III-IV) (BEAT HF II trial, trial No.: NCT03926754). Notably, kidney function is often decreased in HFpEF and HFrEF patients, and chronic heart failure aggravates renal dysfunction mutually. This bidirectional interaction of renal and heart failure

is the key concept in cardiorenal syndrome. Dysfunction of each organ can induce and perpetuate injury in the other via complex hemodynamic, neurohormonal, and biochemical pathways such as over-activated RAAS and sympathetic nervous system, increased nitro-oxidative stress, and inflammatory pathways. Notably, patients with moderately or severely decreased renal function (eGFR<30 or 50 mL/min corresponding to G3b-G5 CKD stages, respectively) were excluded from the Beta3_LVH and BEAT HF II clinical trials. Thus, to our best knowledge, the antiremodeling effects of mirabegron were not investigated in a selected patient population suffering from uremic cardiomyopathy. Considering the exclusion criteria of the aforementioned clinical trials, we aimed to investigate the effects of mirabegron in mild to moderate experimental uremic cardiomyopathy. In our study, mirabegron did not affect the major parameters of renal function, including serum carbamide and creatinine levels, creatinine clearance, and urine volume at the endpoint. However, it had several adverse effects on glomerular function, worsening the proteinuria, renal anemia, serum total cholesterol, and LDL-cholesterol levels in our CKD model. Although mirabegron repressed the LV *IL6* level, white blood cell count remained elevated, suggesting the presence of low-grade systemic inflammation in CKD irrespective of mirabegron-treatment. In our present study, mirabegron could not prevent the development of LVH assessed by M-mode echocardiography and histology in CKD. We speculate that the failing β3-AR overexpression and the β3-AR-coupled activation of eNOS-mediated pathways are responsible for its missing antihypertrophic effects in CKD. Notably, if the β3-AR mRNA expression of only the sham-operated and CKD groups were compared by unpaired t-test, the LV β3-AR expression was significantly increased in the CKD group compared to the sham-operated group. However, the protein level showed no difference between these two groups. This could be explained by i) increased degradation and turnover rate of proteins in CKD as it is considered as a catabolic state, and ii) the β3-AR expression could be regulated time-dependently in uremic cardiomyopathy. We also measured the LV β3-AR mRNA and protein levels at an earlier follow-up time point in the same 5/6 nephrectomy-induced CKD model in male Wistar rats 9 weeks after the operations. At this time, HFpEF is also developed, as we and others described previously. At this earlier follow-up time point, the LV expression of β3-AR mRNA was also significantly increased in the CKD group as compared to the sham-operated group, and there was no significant change in the β3-AR protein levels between the groups, similarly to the week 13 findings. However, it could not be excluded that the β3-AR protein expression might change in a later phase of uremic cardiomyopathy. Notably, in aortic banding-induced heart failure in rats, the cardiac expression of β3-AR mRNA and protein expressions in myocardial

tissues showed a positive correlation with aging and the severity of heart failure. Additionally, the precise mechanisms and functional role of the β3-AR in heart failure induced by different cardiovascular diseases, including diabetes mellitus, acute myocardial infarction, or chemotherapy-induced heart failure forms, are also not fully discovered. Most studies investigating the effects of β3-AR agonists in heart failure demonstrated that β3-AR agonists attenuated cardiac hypertrophy and fibrosis and improved cardiac contractility via coupling of β3-AR to the eNOS/cGMP pathway as the main mechanism. Therefore, we also investigated the LV expression of eNOS, phospho-eNOS, and their ratio in our study. Mirabegron did not increase the total eNOS level and significantly decreased the phospho-eNOS level as compared to the CKD group. Therefore, it seems that the β3-AR is uncoupled from eNOS or unable to activate it, probably, due to elevated nitro-oxidative stress, inflammatory mechanisms, and uremic toxins-induced alterations in CKD. However, in our present study, mirabegron had beneficial effects on DD characterized by e' and E/e', and cardiac fibrosis assessed by histology and LV expressions of *Col1a1*. We hypothesized that mirabegron could have its antiremodeling effects independently of the β3-AR-eNOS-mediated pathway in uremic cardiomyopathy. Interestingly, mirabegron reduced the LV expression of the AT1 receptor in uremic cardiomyopathy. This finding is particularly interesting since the chronic administration of the β3-AR agonists BRL37344 could down-regulate the AT1 receptor expression in the pancreatic and lung tissues of male apoE knock-out mice. Interestingly, a study demonstrated that angiotensin-II administration did not induce cardiac fibrosis and hypertrophy in mice with cardiomyocyte-specific expression of human β3-AR. Recently, others found that chronic infusion of a β3-adrenergic receptor agonist attenuated cardiac fibrosis and improved DD independently of blood pressure in an angiotensin-II-induced heart failure model with hypertension in mice. These results suggest that chronic β3-adrenoceptor activation regulates the expression of angiotensin-II receptor types, and these interactions may play a protective role in the lung, pancreatic, and other tissues such as the heart. The chronic over-activation of the AT1 receptor could play a major role in oxidative stress, inflammation, and ultimately fibrosis in the development of heart failure. Indeed, in our present study, mirabegron significantly reduced the LV expression of the nitro-oxidative stress markers *Nox4* and *Nos2* and the inflammatory marker *IL6* in uremic cardiomyopathy. Our present results are in line with these literature data since mirabegron treatment significantly reduced the AT1 receptor expression in uremic cardiomyopathy, which might lead to its antioxidant, anti-inflammatory, and antifibrotic effects independently of the eNOS/cGMP-mediated pathway in uremic cardiomyopathy. Moreover, the dual antioxidant role of β3-ARs was

described in macrophages: i) β3-AR decreases ROS production by directly inhibiting NADPH-oxidase activity; and ii) β3-AR induces the expression of catalase, a major hydrogen peroxide scavenger. In another study, β3-AR stimulation inhibited cytokine production, including TNF-α and IL6, to prevent myometrial cell apoptosis and extracellular matrix remodeling. However, these secondary antioxidant and anti-inflammatory mechanisms of the β3-AR induction by mirabegron are not well-characterized in HF.

Our present finding on the antiremodeling effects of mirabegron independently of β3-AR-eNOS mediated pathways could be interesting for physicians treating heart failure patients with reduced renal function since mirabegron could have antifibrotic effects in the early stages of uremic cardiomyopathy. Therefore, the involvement of early-stage CKD patients in clinical studies investigating the effects of mirabegron could be considered in the future. However, mirabegron had adverse effects on glomerular function with worsening anemia, proteinuria, and serum cholesterol levels in our CKD model. Therefore, the elevated mortality risk and the possibility of future cardiovascular events might remain high in CKD patients treated by mirabegron. In future studies, the combination of mirabegron with ARBs or cholesterol-lowering drugs such as statins might be rational to potentiate the antiremodeling effects and prevent the adverse effects of mirabegron.

5. Conclusions

The purpose of this research was to evaluate and compare the effects of chronic administration of ARB losartan and β3-AR agonist mirabegron in a rat model of uremic cardiomyopathy. From our results, we can conclude that:

1. Losartan failed to improve the main routine laboratory parameters of kidney function and only showed a mild renoprotective effect.
2. In contrast, mirabegron worsened anemia, proteinuria, and serum cholesterol levels in our CKD model.
3. Losartan could ameliorate LVH, but mirabegron showed no antihypertrophic effects in uremic cardiomyopathy.
4. Both losartan and mirabegron improved DD and cardiac fibrosis.
5. Losartan exerts its protective effects via blocking the AT1 receptor-mediated nitro-oxidative and inflammatory processes as well as eNOS-associated mechanisms in our CKD model.

6. The antifibrotic and anti-inflammatory effects of mirabegron in uremic cardiomyopathy seem to be independent of the β3-AR coupled eNOS-mediated pathways and might be explained by its effect causing repression on the AT1 receptor.
7. Losartan might prevent or markedly slow down the development of uremic cardiomyopathy if it is first administered in the early stages of CKD.
8. The beneficial effects of mirabegron could be used as an "add-on" therapy against cardiac remodeling in uremic cardiomyopathy.

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List of abbreviations

Adrb3: β3-adrenergic receptor (gene)
Agt: angiotensinogen
Agtr1a: angiotensin-II receptor type 1a (gene)
ARB: angiotensin-II receptor blocker
AT1: angiotensin-II receptor type 1a (protein)
β3-AR: β3-adrenergic receptor (protein)
CKD: chronic kidney disease
CVD: cardiovascular disease
Col1a1: collagen type 1 alpha 1 chain
Ctgf: connective tissue growth factor
DD: diastolic dysfunction
eNOS: endothelial nitric oxide synthase
ESRD: end-stage renal disease
GFR: glomerular filtration rate
HE: hematoxylin-eosin
HFpEF: heart failure with preserved ejection fraction
HFrEF: heart failure with reduced ejection fraction
IL1: interleukin-1
IL6: interleukin-6
LV: left ventricular
LVH: left ventricular hypertrophy
Myh6: α-myosin heavy chain
Myh7: β-myosin heavy chain
NO: nitric oxide
Nos2: inducible nitric oxide synthase
NOX: NADPH oxidase
Nox4: NADPH oxidase type 4
Nppa: A-type natriuretic peptide
Nppb: B-type natriuretic peptide
NYHA: New York Heart Association
PLN: phospholamban
Ppia: peptidyl-prolyl isomerase A
PSFG: picrosirius red and fast green
RAAS: renin-angiotensin-aldosterone system
SERCA2a: sarcoplasmic reticulum calcium ATPase 2a
TGF-β: tissue growth factor-beta
Tnf-α: tumor necrosis factor-alpha