

A Nonpeptide, Piperidine Renin Inhibitor Provides Renal and Cardiac Protection in Double-Transgenic Mice Expressing Human Renin and Angiotensinogen Genes

Terry C. Major · Bronia Olszewski · Wendy Rosebury ·
Carlin Okerberg · Tage Carlson · Robert Ostroski ·
Richard Schroeder · Mark C. Kowala · Robert Leadley

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Abstract

Introduction Controlling hypertension by angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB), mechanisms that inhibit later pathway steps in the renin–angiotensin system (RAS), have clinically afforded protection against cardiac and renal disease.

Materials and methods In order to determine if blocking the RAS rate-limiting step of angiotensin II generation via renin inhibition could afford similar end organ protection in a human-relevant preclinical model, this study investigated the cardiac and renal effects of a nonpeptide, piperidine renin inhibitor (RI; 100 mg/kg/day PO) in double transgenic mice (dTGM) which express both human renin and angiotensinogen genes. RI was compared to the ARB, candesartan (3 mg/kg/day PO), and to the ACEI, enalapril

(60 mg/kg/day PO) in a 4-week dosing paradigm. These doses of RI, ACEI and ARB were previously found to normalize mean blood pressure (MBP) to 110+3, 109+7 and 107+6 mmHg, respectively, after 1 day of treatment.

Results and discussion In the dTGM, PRA, plasma aldosterone, GFR, microalbuminuria and left ventricular free wall thickness (LVH) were higher than in the wild type C57BL/6 mice. Microalbuminuria and LVH were significantly reduced by 93% and 9% for the RI, 83% and 13% for enalapril and 73% and 6% for candesartan, respectively. PRA and aldosterone were reduced by the RI 56% and 23%, respectively. These results suggest that the RI provides protection against cardiac and renal disease, similar to ARB and ACEI.

Key words Cardioprotection · Renoprotection · Hypertensive Double Transgenic Mice · Enalapril · Candesartan · Renin Inhibitor · Left Ventricular Hypertrophy and Microalbuminuria

T. C. Major · B. Olszewski · W. Rosebury · R. Ostroski ·
R. Schroeder · M. C. Kowala · R. Leadley
Cardiovascular and Atherosclerosis Biology,
Pfizer Global Research & Development, Pfizer, Inc.,
2800 Plymouth Road,
Ann Arbor, MI 48105, USA

C. Okerberg · T. Carlson
Drug Safety Research and Development,
Pfizer Global Research & Development, Pfizer, Inc.,
2800 Plymouth Road,
Ann Arbor, MI 48105, USA

T. C. Major (✉)
Robert Bartlett Extracorporeal Life Support Laboratory,
Department of General Surgery,
University of Michigan Health System,
1150 W Medical Center Drive,
Ann Arbor, MI 48109, USA
e-mail: tcmajor@umich.edu

The renin–angiotensin system (RAS) is well-established as a major regulator of blood pressure control and renal function through its role in direct vasoconstriction and water/salt homeostasis [1]. Many clinical trials have shown the effectiveness of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) in mitigating the detrimental effects of the major bioactive component of the RAS, namely angiotensin (ANG) II. Ang II contribute to the overall cardiovascular risk of RAS overactivity by promoting hypertension, atherosclerosis and type 2 diabetes [2–5]. Inhibitors of the system, i.e., ACEIs and ARBs, are now standard treatments for hypertension-related organ damage and progressive renal disease as suggested by the clinical trials of Heart Outcome Preven-

tion Evaluation (HOPE) [6–8], Losartan Intervention For Endpoint reduction in hypertension (LIFE) [9, 10], Reduction of Events with Angiotensin Converting Enzyme Inhibition (RENAAL) [11, 12] and Irbesartan Diabetic Nephropathy Trial (IDNT) [13]. The ability of RAS inhibitors to mitigate cardiovascular risk is thought to be mediated, not only by their ability to lower blood pressure, but also by their inhibitory effects on atherosclerosis, left ventricular hypertrophy and renal damage in diabetic patients.

Although ACEI and ARB have been shown to be effective, alternative pathways exist in the RAS which prevent complete blockade of RAS by ACEI and ARB alone. For example, ACE can be bypassed by the serine protease chymase and maybe the major ANG II-forming pathway in the human heart [14]. Also, ANG II is known to act on two receptor subtypes, AT₁ and AT₂. ARBs only block the AT₁ subtype, leaving the AT₂ subtype to elicit its specific pharmacology which is thought to be beneficial. With ACE inhibitors and ARBs not providing complete of blockade of RAS, the rate-limiting and first step in the system, namely, renin catabolizing the specific substrate angiotensinogen to ANG I, provides an optimal target for RAS inhibition. Recently, aliskiren (Tekturna®) has been FDA approved for the US market as a novel monotherapeutic antihypertensive agent but clinical results on cardiac and renal end-organ protection is not yet available [15]. In preclinical models, aliskiren displayed potent and long-lasting BP lowering effects in conscious sodium-depleted marmoset monkeys [16] and recently, in a model of human renin-dependent hypertension, the double transgenic rat harboring the human renin and angiotensinogen genes [17]. Also, aliskiren in the dTGR model was shown to reduce left ventricular hypertrophy and microalbuminuria. Understanding the long-term role renin inhibition has on left ventricular hypertrophy and microalbuminuria as a marker of renal damage could have far reaching impact on this newly available approach to total blockade of the RAS.

To this end, this study investigated the renal and cardiac protective effects of a novel piperidine renin inhibitor at equieffective BP lowering doses to the ACEI, enalapril, and the ARB, candesartan, in the double transgenic mouse model harboring both the human renin and the human angiotensinogen genes. After a 4-week treatment period, the results demonstrate that this piperidine renin inhibitor showed significant improvement in renal and cardiac end organ damage. This improvement was evident when all three RAS inhibitors normalized BP and, thus, compares favorably with end organ protection observed with angiotensin converting enzyme inhibition and angiotensin receptor blockade.

Methods

Materials

The piperidine RI (4-{4-[3-(2-methoxy-benzyloxy)-propoxy]-phenyl}-3-oxo-piperidine-1-carboxylic acid tert-butyl ester) was from the Pfizer Chemistry Department based on the previously published synthetic description [18]. Enalapril maleate was obtained from Sigma Chemical Co. (St. Louis, MO). Candesartan cilextil was purchased from Apin Chemical LTD. (England).

Hemodynamic measurements in experimental mice

The animal handling and experimental procedures were approved by the Pfizer Institutional Animal Care and Use Committee according to NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic mice (3–17 months of age) carrying both the human renin and human angiotensinogen genes were generated and obtained from the Dr. Curt Sigmund laboratory (Univ. of Iowa, Iowa City, IA). Briefly, the double transgenic mice (dTGM) were generated by breeding heterozygous human renin transgenic mice with heterozygous human angiotensinogen transgenic mice. Human REN 9 transgenic female mice with the human renin gene were mated with human angiotensinogen transgenic male mice line 204/1 to generate the double transgenic mouse. The details of the double transgenic mouse generation and phenotype were previously described [19]. Breeding was conducted at Charles River Laboratories (Wilmington, Massachusetts). The transgenic line was developed on a C57 BL/J wildtype background. The dTGM were utilized for hemodynamic parameter monitoring using the radiotelemetry system from Data Sciences International and Ponemah/Gould (St. Paul, MN). The catheters were placed in the left carotid artery and advanced to the aortic arch. To allow for recovery following surgery, the mice were not put on study for a minimum of 7 days. The mice were fed LabDiet 5001® (LabDiet, Division of Purina Mills, Richmond, Indiana). Animals were allowed food and water ad libitum.

The radiotransmitters provided a continuous measurement of the aortic blood pressure from which Ponemah (Gould Instruments Inc, Valley View, Ohio) software derived mean arterial blood pressure (MABP) and heart rate (HR) values. One-minute averages were summarized at 15-min intervals using an Excel (Microsoft Corporation, Redmond, Washington) macro developed within Pfizer Ann Arbor Laboratories.

Dose selection studies

In order to find doses of the three RAS inhibitors that lower BP equally, preliminary single dose response studies were

performed for the RI, the ACEI, enalapril, and the ARB, candesartan. The dose range for the RI was 30 and 100 mg/kg with 6 dTGM for each dose. Enalapril was tested at doses 3–60 mg/kg ($n=6/\text{dose}$) and candesartan at 1–10 mg/kg ($n=6/\text{group}$). From these studies the doses and dosing intervals selected for the RI, enalapril and candesartan were 100 mg/kg, 60 mg/kg and 3 mg/kg, respectively. The oral vehicle for the RAS inhibitors was 3% dimethyl acetamide plus 97% sulfobutylether-beta-cyclodextrin (40% w/v in 50 mM tris base), which had no hemodynamic effects. The protocol to assess blood pressure and heart rate *via radiotelemetry* for each RAS inhibitor is as follows. On day 0, a baseline blood pressure was established in each mouse over a 20-h period. The compound treatment via oral gavage was administered on day 1. The dosing regimen indicated above for the RI, enalapril or candesartan was then used for the non-radiotelemetered dTGM 4-week studies that evaluated the renal and cardiac end organ damage. The total dTGM used in this end organ damage evaluation was 36 in the vehicle group, 14 in the RI group, 26 in the enalapril group and 23 in the candesartan group. Due to previous experience with radiotelemetry implants showing slight renal inflammation which would confound potential organ protection data interpretation, the subsequent 4-week long end organ damage studies were not done with implants. Space restrictions prevented two separate groups of animals for both 4-week hemodynamic evaluation as well as the end organ protection studies.

Plasma renin activity (PRA) and plasma aldosterone

Mouse blood (0.5–1 ml) was drawn via cardiac stick under isoflurane gas anesthesia into EDTA-containing vacutainer tubes (Beckton-Dickinson, Rutherford, NJ) at room temperature and then the animal was euthanized. The tubes were centrifuged $2,083\times g$ at room temperature. The plasma was quickly collected as 80 μl aliquots for PRA measurement and 200 μl aliquots for plasma aldosterone determination. The plasma samples were then stored at -80°C . Transgene human PRA in mouse plasma was measured by radioimmunoassay using high-affinity antibodies trapping generated Ang I which was first described by Poulsen and Jorgensen [20] and modified by Nussberger et al. [21]. Briefly, into a chilled rabbit antihuman angiotensin I coated 12×75 mm tube (DiaSorin, Stillwater, MN) on ice, 75 μl plasma, control, or angiotensin I standard (both from DiaSorin, Stillwater, Minnesota) was added. Seven microliters (7 μl) of Tris–EDTA buffer containing 0.2 M Tris (Bio-Rad, Hercules, CA) plus 200 mM EDTA (Aldrich, Milwaukee, WI) and 3 μl Tris albumin buffer containing 0.2 M Tris, 3 g/L human albumin Fraction V (Sigma-Aldrich, Steinheim, Germany) were also added to the tubes.

Tubes were centrifuged at $916\times g$ and incubated at 37°C for 60 min then placed in an ice bath. Seventy-five microliters of low renin plasma was added to the angiotensin I standards in order to match unknown sample protein level and an additional 75 μl of Tris–albumin buffer was added to the unknown samples on ice. One milliliter (1 ml) of [^{125}I] labeled angiotensin I standard in a reaction buffer (DiaSorin, Stillwater, MN) was added to all the tubes, which were then incubated for 20 to 24 h at 4°C . At the end of the incubation, tubes were aspirated and counted in a Wallac gamma radiation counter (PerkinElmer, Wellesley, MA). A standard curve for human renin activity was generated in the concentration range of 0.2 to 100 ng/ml. The limit of detection is 0.20 ng of Ang I per mL per hour when 75 μl of plasma were analyzed.

In determining the potency of the renin inhibitor to antagonize human renin, a modified *in vitro* PRA assay was setup which utilized a recombinant human renin (JP-2 clone; Jeanette Peevers, Cardiovascular Molecular Science, PGRD, Ann Arbor, Michigan) with 250 $\mu\text{IU}/\text{ml}$ (International Units) of activity. Briefly, into a chilled rabbit antihuman angiotensin I coated 12×75 mm tube (DiaSorin, Stillwater, MN) on ice, 75 μl low renin human plasma, control, or angiotensin I standard (both from DiaSorin, Stillwater, Minnesota) were added. Seven microliters (7 μl) of Tris–EDTA buffer containing 0.2 M Tris (Bio-Rad, Hercules, CA) plus 200 mM EDTA (Aldrich, Milwaukee, WI) and 3 μl Tris albumin buffer containing 0.2 M Tris, 3 g/L human albumin Fraction V (Sigma-Aldrich, Steinheim, Germany) were then added to the tubes. The recombinant human renin was then added at 0.51 μl . Increasing concentrations of the piperidine renin inhibitor or DMSO vehicle were then added to the tubes. The tubes were then centrifuged at $916\times g$ and incubated at 37°C for 60 min. Following the incubation, the tubes were immediately placed in an ice bath to stop the conversion of angiotensinogen to Ang I. Low renin plasma (75 μl) was added to the tubes containing the angiotensin I standards as described above and an additional 75 μl of Tris–albumin buffer was added to the rest of the samples which were on ice. One milliliter (1 ml) of [^{125}I]–labeled angiotensin I standard in a reaction buffer (DiaSorin, Stillwater, MN) was added to all the tubes, which were then incubated for 20 to 24 h at 4°C . At the end of the incubation, tubes were aspirated and counted in a Wallac gamma radiation counter (PerkinElmer, Wellesley, MA).

In determining plasma aldosterone levels, a radioimmunoassay utilizing [^{125}I]–labeled aldosterone that competes for a fixed time with aldosterone in the plasma sample for aldosterone-specific antibody immobilized to polypropylene tube walls was done. The polyclonal rabbit antibody against human aldosterone has been shown to have a high

specificity for aldosterone (100%) compared to other steroids such as 11-deoxycorticosterone (0.006%) [22]. Briefly, the previously frozen 200 μ l plasma samples were thawed for plasma aldosterone determination. Into an antihuman aldosterone-coated 12 \times 75 mm tube (Coat-A-Count, Siemens Medical Solutions Diagnostics, Tarrytown, NY) at room temperature (RT), 200 μ l plasma, control, or aldosterone standard were added to appropriate tubes. One microliter of [125 I]-labeled aldosterone was added to every tube and then incubated for 18 h at RT. At the end of the incubation, tubes were thoroughly aspirated and counted in a Wallac gamma radiation counter (PerkinElmer, Wellesley, MA) for 1 min. A standard curve for aldosterone was generated in the concentration range of 25 to 1,200 pg/ml with an assay sensitivity of 11 pg/ml.

Microalbuminuria assay and biochemical determinations

A direct competitive ELISA was utilized for measuring mouse urinary albumin (Albuwell M, Exocell Inc., Philadelphia, PA) at the end of the 4-week treatment period. To complete the assay, 50 μ l of controls, standards or diluted mouse *urine* samples (1:13) plus 50 μ l of rabbit anti-mouse albumin antibody were added to a 96 well plate which is coated with murine albumin and allowed to incubate for 30 min at room temperature. After washing the plate, an anti-rabbit HRP conjugate (100 μ l) was then added and allowed to react for 30 min at room temperature. Subsequently, the plate is washed, and unbound reactants are washed away. Only the antibody-conjugate bound to the albumin in the stationary phase remains. A chromogenic substrate, TMB, is added to the wells, and color development ensues in the next 5–10 min. Color intensity is inversely proportional to the logarithm of the mouse albumin concentration in the plasma sample. This assay has a useful dynamic range of 0.313–10 μ g mouse albumin/ml, and the samples were diluted 1:13 to fall within that range. In order to normalize the urinary albumin levels, urinary creatinine was measured using the creatinine aminohydrolase/hydrogen peroxide method [23] with detection at 670 nm (Companion Creatinine kit, Exocell Inc., Philadelphia, PA). In separate samples, sodium, potassium and BUN were measured in serum and urine using a Vitros 250 automated chemistry analyzer (Ortho Clinical Diagnostics). For the measurements of sodium, potassium and BUN, urine samples were diluted 5-fold prior to analysis. Sodium and potassium were measured by direct potentiometry and BUN was measured in serum by the urease/ammonia method with detection at 670 nm. Osmolality was measured with a Precision Systems Multi-Osmette Model 2430 Osmometer (Precision Systems, Inc.) using a freezing-point depression method. Glomerular filtration rate (GFR) was estimated from the calculation of body weight-

adjusted creatinine clearance using the formula: $GFR = \frac{\text{urine creatinine (mg/dl)} \times \text{urine volume (ml/minute)}}{\text{serum creatinine (mg/dl)} \times \text{body weight (kg)}}$. Creatinine clearance has been shown to be a reliable measure of GFR [24, 25].

Left ventricular hypertrophy measurement by cardiac ultrasound

At the end of the 4-week treatment period, an echocardiograph assessment was performed under light isoflurane anesthesia and with spontaneous respiration using a face mask. The mice were positioned on their left side on a heating pad to maintain body temperature. A trans-thoracic short-axis two-dimensional image of the left ventricle was taken at the level just below the papillary muscle using a Hewlett-Packard Sonos 5500 echocardiograph machine (Hewlett-Packard Palo Alto, CA) and a 15-MHz high-resolution transducer (Agilent Technologies, Novi, MI). Left ventricular free wall thickness was measured at end systole, and was determined from the average of 3 consecutive cardiac cycles. After the echocardiograph measurements, the animals were weighed for total body weight (g) then sacrificed. The hearts were removed, excess fluid removed and wet weights (g) measured. The ratio of heart weight to body weight was calculated as an index of cardiac hypertrophy.

Statistical analysis

Data are expressed as mean \pm SEM. Comparisons between C57BL/J wildtype and the vehicle-treated dTGM were analyzed by Student's *t*-test. The comparison between the dTGM vehicle at the time of the maximal hemodynamic change for each RAS inhibitor was also analyzed by Student's *t*-test. A one-way ANOVA with Hsu's mean comparison [26] was used to compare means of dTGM vehicle, the renin inhibitor, enalapril and candesartan treatment groups. Values of $P < 0.05$ were considered statistically significant.

Results

In vitro PRA

The nonpeptide, piperidine, RI (4-{4-[3-(2-methoxybenzyloxy)-propoxy]-phenyl}-3-oxo-piperidine-1-carboxylic acid tert-butyl ester) has a structure shown in Fig. 1 A. As shown in Fig. 1 B, the RI had a potent effect in inhibiting recombinant human renin with an IC_{50} value of 26 ± 7 nM in the in vitro PRA using recombinant human renin. The inhibitor is specific in its ability to antagonize human renin

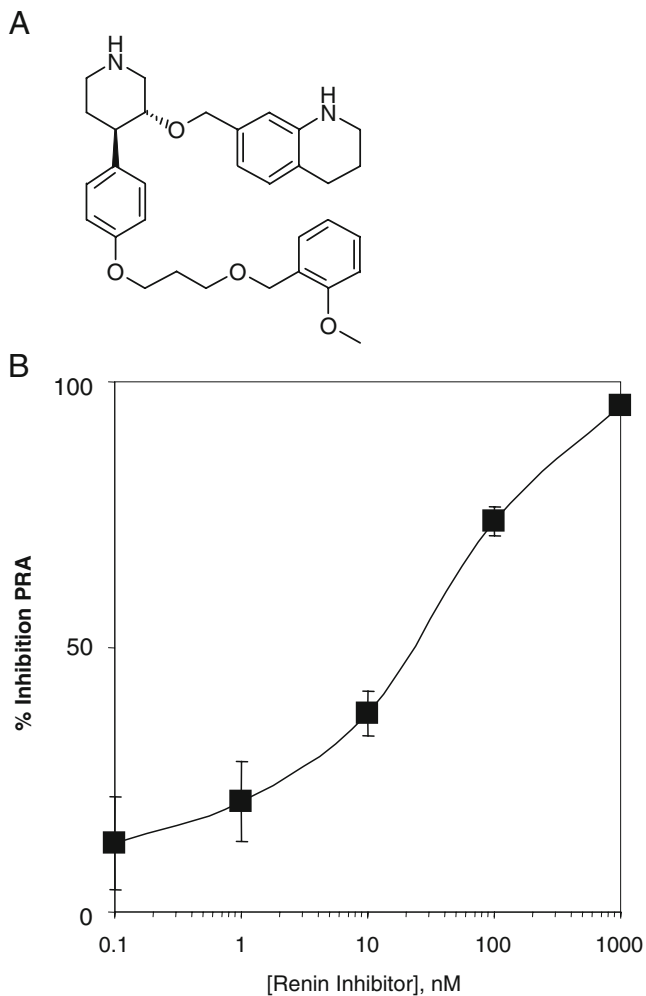


Fig. 1 Effects of Nonpeptide, Renin Inhibitor, on in vitro Plasma Renin Activity (PRA) in Human and Rat Plasma. **a** Structure of the nonpeptide piperidine RI. **b** Using an antibody trapping method to assess the in vitro inhibition of recombinant human renin activity by RI, a percent inhibition curve was constructed with increasing concentrations of RI. Data are means+SEM

because the RI was less potent in inhibiting endogenous rat renin activity which had an IC_{50} value of 10,000 nM.

Blood pressure in dTGM

To establish equal BP lowering efficacy for the three RAS inhibitors, RI, enalapril and candesartan, separate single dose–response experiments were performed. In these studies, the dosing regimen was 100 mg/kg for the RI, 60 mg/kg for enalapril and 3 mg/kg for candesartan for 1 day. Shown in Table 1 are the maximal hemodynamic responses to each RAS inhibitor in a 20 hour treatment period. The RI was equally efficacious to the ACEI, enalapril, and the ARB, candesartan, in lowering mean BP at the doses chosen as compared to the vehicle-treated dTGM (103 ± 6 , 96 ± 8 and 94 ± 5 mmHg, respectively, compared to vehicle, 149 ± 7 ; $p<0.05$). The C57 wildtype

mice had a mean BP of 108 ± 1 mmHg over a 20-h period indicating that all three RAS inhibitors normalized mean BP in the dTGM. As documented by many RAS inhibitors, heart rate was not affected by RI, enalapril or candesartan in the dTGM. Both systolic and diastolic BP were lowered equally (about 50 mmHg) with the RI compared to ACEI and ARB. The duration of significant BP lowering for the RI and enalapril were approximately 10–12 h (data not shown). Since a total daily dose of 100 mg/kg produced a comparable mean BP fall as enalapril and candesartan, the RI dosing regimen for the subsequent 4-week end organ damage study utilized two 50 mg/kg oral doses given 8–10 h apart in order to maintain BP lowering efficacy for a greater portion of 24 h. Therefore, for the subsequent 4 week end organ damage study, doses were selected based on the equieffective BP lowering and were 50 mg/kg BID RI, 30 mg/kg BID enalapril and 3 mg/kg QD candesartan.

RAS inhibitor effects on PRA and aldosterone levels

To determine the effects of the RI, enalapril or candesartan on the RAS, we evaluated PRA and plasma aldosterone levels in the dTGM after 4 weeks of treatment with these agents. The dTGM plasma had a significant elevation in PRA compared to the C57 wildtype mouse (Fig. 2 A). All three RAS inhibitors produced statistically significant decreases in PRA ($p<0.05$). The decreases in PRA after 4 weeks of RI, enalapril or candesartan treatment in the dTGM were 56, 35 and 33%, respectively (Fig. 2 A). The observed decrease with both enalapril and candesartan in the dTGM may be due to an inherent artifact of the PRA assay since the PRA assay is specific to human renin and angiotensinogen substrate conversion to Ang I.

Plasma aldosterone levels were also significantly increased over the C57 wildtype mice as shown in Fig. 3 B (511 ± 76 pg/ml dTGM versus 187 ± 31 pg/ml C57Bl/J, $p<0.05$). After 4 weeks of treatment, enalapril and candesartan, but not the RI, significantly decreased plasma aldosterone levels ($p<0.05$). As shown in Fig. 2 B, the decreases in plasma aldosterone to the RI, enalapril and candesartan-treated groups were 23, 51 and 67%, respectively.

Renin inhibitor effects on cardiac end organ damage

The RI was compared to the ACEI, enalapril and the ARB, candesartan, to determine if it could protect the heart and kidneys from end organ damage after 4 weeks of treatment. In evaluating cardiac end organ damage, the heart to body weight ratio and left ventricular hypertrophy via echocardiography were determined. Double transgenic mice (3–13 months of age) had significant increases in the heart/body weight ratio compared to the C57 wildtype mice (0.0075 ± 0.0004 vs. 0.00463 ± 0.00024 ; $p<0.05$). This

Table 1 Comparable blood pressure lowering by the renin inhibitor, enalapril and candesartan in double transgenic mice in a 20-h treatment period

Parameter	Vehicle	Renin inhibitor (100 mg/kg/day)	Enalapril (60 mg/kg/day)	Candesartan (3 mg/kg/day)
Mean blood pressure (mmHg)	149±7	103±6*	96±8*	94±5*
Systolic blood pressure (mmHg)	171±8	119±7*	111±8*	108±5*
Diastolic blood pressure (mmHg)	131±7	86±6*	82±9*	83±5*
Heart rate (BPM)	537±16	536±28	462±92	508±75

Values are means±SEM, $n=6$.

* $p<0.05$ versus vehicle group.

increase in heart weight in the dTGM became manifest in the left ventricular free wall thickness as measured by 2-dimensional echocardiography. Figure 3 shows a significant ($p<0.05$) increase in left ventricular free wall thickness in the dTGM over its age-matched C57 wildtype counterpart

(1.16 ± 0.02 vs. 0.77 ± 0.01 μm). After 4 weeks of treatment, the left ventricular free wall thickness was significantly ($p<0.05$) reduced by the RI, enalapril or candesartan compared to the vehicle control (Fig. 3). All three RAS inhibitors reduced left ventricular free wall thickness to a statistically similar extent ($p<0.05$).

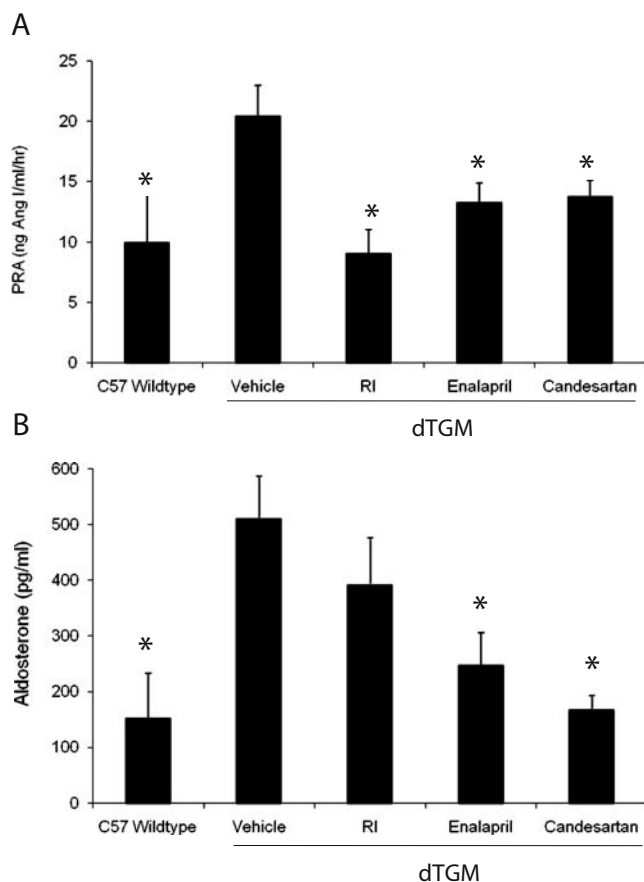


Fig. 2 Effects of the Renin Inhibitor, Enalapril or Candesartan on Plasma Renin Activity and Plasma Aldosterone in Double Transgenic Mice. **a** The effects of vehicle ($n=3333$), 100 mg/kg/day RI ($n=9$), 60 mg/kg/day enalapril ($n=22$) or 3 mg/kg/day candesartan ($n=22$) on dTGM PRA compared to untreated C57 wildtype ($n=6$) as measured in the Ang I antibody trapping method for endogenous renin activity. **b** The effects of vehicle ($n=28$), RI ($n=9$), enalapril ($n=26$) or candesartan ($n=23$) on dTGM plasma aldosterone compared to untreated C57 wildtype ($n=6$) as measured in the RIA for aldosterone. The results are expressed as means±SEM. * $p<0.05$ vs. dTGM vehicle group using one-way ANOVA

Renin inhibitor effects on renal end organ damage

Microalbuminuria, glomerular filtration rate (GFR), urine sodium and potassium were measured to determine the renoprotective effects of the RI as compared to the ACEI, enalapril, and the ARB, candesartan. As shown in Fig. 4, microalbuminuria, which was normalized to urine creatinine levels, was significantly elevated in the 4 week vehicle-treated dTGM compared to age-matched C57 wildtype mice (809.3 ± 160.8 vs. 6.8 ± 1.1 mg albumin/g creatinine; $p<0.05$). The RI significantly decreased microalbuminuria from the vehicle control level to 60.1 ± 20 mg

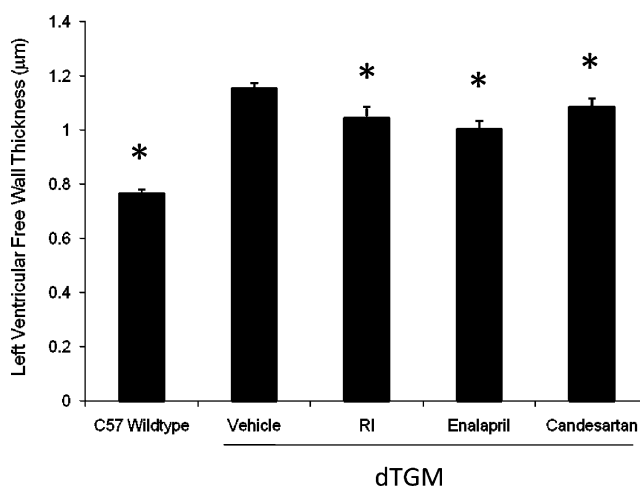


Fig. 3 Effects of renin inhibitor, enalapril and candesartan on left ventricular hypertrophy in double transgenic mice. The effects of vehicle ($n=36$), 100 mg/kg/day RI ($n=14$), 60 mg/kg/day enalapril ($n=24$) or 3 mg/kg/day candesartan ($n=11$) on dTGM left ventricular free wall thickness compared to untreated C57 wildtype ($n=6$) as measured with two-dimensional echocardiography. The results are expressed as means±SEM. * $p<0.05$ vs. dTGM vehicle group using one-way ANOVA

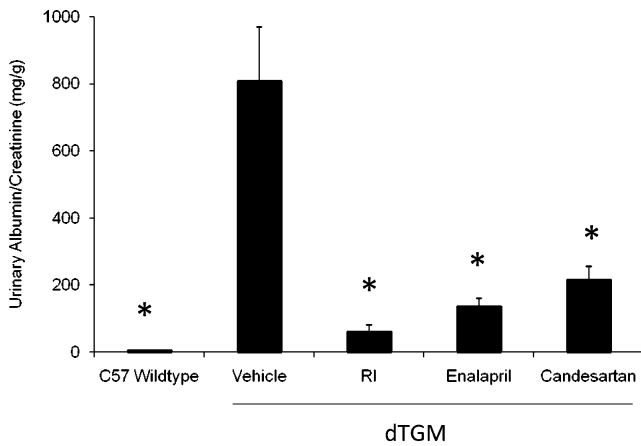


Fig. 4 Effects of renin inhibitor, enalapril and candesartan on microalbuminuria in double transgenic mice. Effects of vehicle ($n=29$), 100 mg/kg/day RI ($n=7$), 60 mg/kg/day enalapril ($n=11$) or 3 mg/kg/day candesartan ($n=13$) on ratio of urinary albumin/creatinine after 4 weeks of treatment in dTGM compared to untreated C57 wildtype mice ($n=7$). The results are expressed as means \pm SEM. * $p<0.05$ vs. dTGM vehicle group using one-way ANOVA

albumin/g creatinine after the 4 weeks of treatment (Fig. 4). This was a 93% reduction in the albumin level in the urine of the RI-treated dTGM compared to the vehicle control. For comparison, the ACEI, enalapril, and the ARB, candesartan, decreased microalbuminuria after 4 weeks of treatment compared to the vehicle control (137 ± 23 and 217.7 ± 39 mg albumin/g creatinine, respectively; $p<0.05$). The percent reduction in microalbuminuria for the RI was similar to that observed for either enalapril (83% decrease) or candesartan (73% decrease) even when all three RAS inhibitors had comparable BP lowering. In addition, GFR was significantly reduced and within the normal values observed in the C57 wildtype mice compared to the vehicle-treated dTGM (Table 2). Also shown in Table 2, the RI, enalapril or candesartan markedly increased the levels of urinary sodium and potassium excretion (mmol/L) which increased urine osmolality (mOsm/kg urine) after 4 weeks of compound treatment in the dTGM.

Discussion

Understanding the role renin inhibition has in preventing end organ damage will provide important insights into whether or not this mechanism provides additional or complementary benefits over other RAS antagonists, such as ACEI and the ARBs. This study demonstrated that the small molecule RI can normalize BP, attenuate cardiac hypertrophy and decrease the renal marker for kidney disease, microalbuminuria in the dTGM which expresses human components of the RAS. In the current study, the RI reduced BP in one day to a similar level as the RAS inhibitors, enalapril and candesartan. In the face of

Table 2 Effects of the nonpeptide renin inhibitor, as compared to the ACEI, enalapril or the ARB, candesartan, on cardiac and renal functional parameters in dTGM

Parameter	C57 Wildtype Mice		dTGM						
	Baseline		Vehicle	Renin Inhibitor (100 mg/kg/day)	% change-RI	Enalapril (60 mg/kg/day)	% change- enalapril	Candesartan (3 mg/kg/day)	% change- candesartan
Heart wt/Body wt ratio	0.00463 \pm 0.00024		0.0075 \pm 0.00004	0.0063 \pm 0.0003*	-16	0.0051 \pm 0.00016*	-32	0.00533 \pm 0.00028*	-29
Lt. kidney/Body wt ratio	0.00586 \pm 0.00016		0.0065 \pm 0.0001	0.0067 \pm 0.0002	3	0.00687 \pm 0.0002	6	0.00661 \pm 0.0002	2
Rt. kidney/Body wt ratio	0.00624 \pm 0.00024		0.0069 \pm 0.0001	0.0071 \pm 0.0002	3	0.0074 \pm 0.00059	7	0.00625 \pm 0.00016	-9
GFR (ml/min/kg)	7.11 \pm 1.00		9.97 \pm 0.65	6.15 \pm 0.61*	-38	6.12 \pm 0.91*	-39	6.29 \pm 0.59*	-37
U_{Na} (mmol/L)	115.3 \pm 11.9		98.9 \pm 11.5	116.3 \pm 8.7	18	160.3 \pm 15.6*	62	151.0 \pm 13.0*	53
U_K (mmol/L)	328.2 \pm 35.9		175.1 \pm 15.6	286.7 \pm 28.1*	64	269.0 \pm 17.0*	54	279.2 \pm 21.9*	59
$U_{osmolality}$ (mosm/mg)	ND		1235 \pm 178	1523 \pm 150	23	1923 \pm 157*	56	1978 \pm 154*	60

Values are means \pm SEM

ND Not determined

* $p<0.05$ versus dTGM vehicle group using ANOVA

comparable BP lowering by the RAS inhibitors in the dTGM, which has BP 50–60 mmHg higher than their wildtype counterparts, the major finding was that RI was as effective as the ACEI and ARB in protecting the heart from left ventricular hypertrophy and the kidney from microalbuminuria. Our results provide direct evidence in an established model of Ang II-induced end organ damage and high BP that a small molecule RI can protect the heart and kidney from the damaging effects of Ang II as efficaciously as enalapril or candesartan.

Renin inhibitor development has progressed significantly over the last few years. The earliest RIs, which were stable peptide-like analogues of the renin substrate, angiotensinogen, were as effective in reducing BP in salt-depleted marmosets when compared to ACEIs [16, 27]. However, these RIs had poor bioavailability, had short duration of action and were not suitable for clinical use due to lack of oral bioavailability. Marki et al. reported a novel, non-peptidic renin inhibitor (RI) of the piperidine type that was synthetically optimized and was much improved in potency as well as physicochemical and metabolic properties over the earlier peptidomimetic renin inhibitors [18]. Our results confirm the data from Marki et al. that demonstrated this piperidine RI lowered BP and also normalized microalbuminuria and kidney end organ damage in double transgenic rats harboring both the human angiotensinogen and renin genes. In addition, we found that the piperidine RI reduced the left ventricular free wall thickness which is a marker of cardiac hypertrophy. The RI was dosed for 4 weeks in each model resulting in reduced renal and cardiac end organ damage that was equivalent between the two hypertensive models. One of the limitations with the dTGR, however, is the rate that these rats develop mortality. The rapid rate of pathological development in the dTGR [28, 29] may not represent the chronic development of end organ damage as seen in the human condition of renin-dependent hypertension. The dTGM, on the other hand, develop end organ damage in a similar pattern that is seen in humans and may lend itself as a more appropriate model for RAS-mediated end organ damage [19, 30–32]. Even with the differences in the transgenic animal models, it appears that renin inhibition is an effective mechanism of attenuating cardiovascular disease in that it inhibits the rate-limiting step of the RAS.

The recently FDA-approved RI, aliskiren, has been shown to be effective in inhibiting human renin activity with an IC_{50} value of 0.6 nM [33]. Even though the piperazine RI has an IC_{50} value of 26 nM against human renin in vitro as compared to aliskiren's 0.6 nM, the advantage for the piperazine RI is in the significantly reduced chemical synthetic path compared to the synthetic path for aliskiren. Preclinically, aliskiren has been shown to be effective in normalizing BP in salt-depleted marmosets

(high plasma rennin model) and spontaneously hypertensive rats [16]. Aliskiren has also been shown to be as effective in normalizing BP as ARBs such as losartan at similar doses (37.5, 75, 150, 300 mg/day aliskiren vs. 100 mg/day losartan) [34] or irbesartan (150 mg)[35] in hypertensive patients. In addition to the BP lowering, renin inhibition by aliskiren has been shown to compare favorably to angiotensin receptor blockade in reversing organ damage in the dTGR [17]. Aliskiren is the only renin inhibitor that has shown BP lowering efficacy in patients but long-term effects such as normalization of albuminuria and kidney tissue damage are still pending [15]. Recently, Parving et al. showed that the combination of 300 mg/kg aliskiren with 100 mg/kg losartan treatment showed an additional 20% reduction in the urinary albumin/creatinine ratio compared to placebo/losartan treatment after 3 months. The additional anti-proteinuria effect of the RI on top of the ARB effect was independent of any further BP lowering [36].

The marked reductions in microalbuminuria with modest effects on systemic BP lowering may indicate that the piperidine RI may accumulate in the kidneys. The direct measurement of the RI in kidney tissue was not done in this study. Evidence, however, in support of this hypothesis has been shown in the results of Richter et al. where the non-peptide RI, remikiren, had high concentrations in the kidneys of squirrel monkeys. This may explain the high pharmacological activity of remikiren despite the high plasma clearance of the drug [37].

In addition to the protective effects of the piperidine RI on left ventricular wall thickness and microalbuminuria, the primary markers of cardiac hypertrophy and renal dysfunction, respectively, shown in our study, the RI had a tendency to attenuate the increase in plasma aldosterone levels in the dTGM. The interesting point here is that an association appears between plasma aldosterone levels and the degree of microalbuminuria. In our study, there is a decrease in both plasma aldosterone and the level of microalbuminuria with the piperidine RI. In support of this observation, one study [38] showed that the aldosterone antagonist, eplerenone, markedly attenuated proteinuria in SHRSP and concluded that aldosterone acts as a primary mediator of RAS-driven renal damage. The important new findings in this report are that (1) the piperidine RI is effective in normalizing BP and (2) the RI can protect the heart from hypertrophy and the kidneys from microalbuminuria equally with enalapril and candesartan. The piperidine RI when compared to the other nonpeptide RI, aliskiren, has a similar efficacy in both as an antihypertensive and protector of end organ damage but has a significant advantage in that the piperidine RI requires less than half of the chemical synthetic steps than aliskiren [27]. This advantage would help reduce the cost of goods and promote a more cost effective antihypertensive therapy.

One of the limitations of this study is that BP was not followed out the full 4 weeks of RI treatment. However, preliminary work with the ACEI, enalapril, and the ARB, candesartan, had shown that 7 day dosing showed no change in dTGM BP compared to the fall in the first day of treatment and thus we assumed the RI would also continue to maintain a normalized BP. The effects of 4 weeks of RI treatment on BP remains to be investigated. Also, due to technical problems and deaths, not all the dTGM were available for plasma renin activity, plasma aldosterone, left ventricular hypertrophy and microalbuminuria testing. However, statistical power was still maintained to demonstrate equivalence between the RAS inhibitors.

In summary, the novel piperidine RI is an effective, species specific, RAS inhibitor that normalizes BP and protects both the heart and kidney from hypertrophy and microalbuminuria, respectively, in a hypertensive transgenic mouse model expressing human genes for angiotensinogen and renin. The effects of the RI were comparable to the precedented RAS inhibitors, ACEI and ARB. The data indicate that renin inhibition with this new piperidine RI could provide a valuable and effective addition to antihypertensive treatments which block the renin–angiotensin system.

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