Direct Angiotensin II Type 2 Receptor Stimulation in N\textsuperscript{o}-Nitro-L-Arginine-Methyl Ester–Induced Hypertension
The Effect on Pulse Wave Velocity and Aortic Remodeling

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Abstract—Pulse wave velocity (PWV), a direct marker of arterial stiffness, is an independent cardiovascular risk factor. Although the angiotensin II type 1 receptor blockade belongs to major antihypertensive and cardioprotective therapies, less is known about the effects of long-term stimulation of the angiotensin II type 2 receptor. Previously, compound 21, a selective nonpeptide angiotensin II type 2 receptor agonist improved the outcome of myocardial infarction in rats along with anti-inflammatory properties. We investigated whether compound 21 alone or in combination with angiotensin II type 1 receptor blockade by olmesartan medoxomil could prevent PWV increase and aortic remodeling in N\textsuperscript{o}-nitro-L-arginine-methyl ester (L-NAME)–induced hypertension. Male adult Wistar rats (n=65) were randomly assigned to control, L-NAME, L-NAME+compound-21, L-NAME+olmesartan, and L-NAME+olmesartan+compound-21 groups and treated for 6 weeks. We observed that L-NAME hypertension was accompanied by enhanced PWV, increased wall thickness, and stiffness of the aorta, along with elevated hydroxyproline concentration. Olmesartan completely prevented hypertension, PWV and wall thickness increase, and the increase of aortic stiffness and partly prevented hydroxyproline accumulation. Compound 21 partly prevented all of these alterations, yet without concomitant prevention of blood pressure rise. Although the combination therapy with olmesartan and compound 21 led to blood pressure levels, PWV, and wall thickness comparable to olmesartan-alone–treated rats, only in the combination group was complete prevention of increased hydroxyproline deposition achieved, resulting in even more pronounced stiffness reduction. We conclude that chronic angiotensin II type 2 receptor stimulation prevented aortic stiffening and collagen accumulation without preventing hypertension in rats with inhibited NO synthase. These effects were additive to angiotensin II type 1 receptor blockade, yet without additional blood pressure–lowering effect, and they seem to be NO and blood pressure independent. (Hypertension. 2012;59[part 2]:485-492.)

Key Words: L-NAME ■ vascular remodeling ■ arterial stiffness ■ pulse wave velocity ■ renin-angiotensin system ■ hypertension

Arterial hypertension is a highly prevalent and still insufficiently controlled medical condition leading to severe cardiovascular complications.\textsuperscript{1,2} One of the main contributors to the cardiovascular risk in these patients is the associated target organ damage. Augmented pulse wave velocity (PWV), which is considered to be the best and direct marker of arterial stiffness,\textsuperscript{3} is regarded as an indicator of subclinical organ damage by current European guidelines for hypertension treatment.\textsuperscript{4}

The effects of current antihypertensive treatment on PWV have been investigated with various outcomes. The angiotensin II type 1 receptor (AT\textsubscript{1}R) antagonists, together with angiotensin-converting enzyme inhibitors, belong to the gold standard cardioprotective therapies.\textsuperscript{5,6} In a study on 113 hypertensives, 2- to 3-year treatment with candesartan produced a larger reduction of brachial-ankle PWV than treatment with anlolidipine,\textsuperscript{7} and eprosartan in contrast to atenolol effectively reduced PWV despite the same level of blood pressure (BP) control in subjects with never-treated hypertension.\textsuperscript{8} Anti-AT\textsubscript{1}R–based therapy was superior over treatment not containing an AT\textsubscript{1}R antagonist in preventing PWV increase in patients with essential hypertension,\textsuperscript{9} and high-dose valsartan treatment achieved more pronounced brachial-ankle PWV reduction than low-dose valsartan+diopter despite a similar level of BP reduction in patients with morning hypertension.\textsuperscript{10} On the other hand, in the REASON (pReterax in regression of Arterial Stiffness in a controled double-blNd study) Project, both the combination of subtherapeutic doses of perindopril and indapamide or a full dose of atenolol reduced slightly but significantly PWV,
yet without any difference between the 2 treatment regimens. In a comparison of 4 major antihypertensive therapies in patients with systolic hypertension, none of the medications (perindopril, atenolol, lercanidipine, or bendrofluazide) was able to reduce PWV, and a 4-week treatment with atenolol reduced PWV in hypertensive patients, whereas perindopril did not. It seems that BP lowering might not always lead to PWV reduction, especially when structural vascular alterations are already present, and a failure to attenuate aortic stiffness along with BP lowering had a negative impact on the survival of patients with end-stage renal failure. Thus, there is currently an unremitting search for novel agents with destiffening properties without or even beyond BP-lowering effects.

Despite the established role of AT,R antagonists in the treatment of various cardiovascular conditions, less is known about the possible vasculoprotective effects of direct stimulation of the angiotensin II type 2 receptor (AT2R). AT2R was already reported to exert antiproliferative, anti-inflammatory, and cardioprotective effects that at least partly counterbalance the effects of AT,R stimulation. In vivo inhibition of NO synthase by treating animals with Nω-nitro-L-arginine-methyl ester (L-NAME) provides a well-established model of hypertension, vascular wall remodeling, and PWV increase. Using this model, we aimed to investigate the effects of long-term direct AT2R stimulation by the novel nonpeptide AT2R agonist, compound 21, on aortic remodeling and PWV in L-NAME–treated rats. Furthermore, we aimed to compare the effects of AT2R stimulation to the effects of the AT1R antagonist, olmesartan, and to evaluate the effects of the combination treatment with compound 21 and olmesartan.

### Methods

#### Animals and Treatment

Male 10-week–old Wistar rats (Janvier, Le Genest-St-Ise, France) were randomly assigned into 6 groups treated with vehicle (Ctrl; n = 15), 50 mg/kg per day of L-NAME (n = 15); L-NAME and 0.3 mg/kg per day of compound 21 (LN + C21; n = 15); L-NAME and 0.3 mg/kg per day of olmesartan medoxomil (LN + Olm; n = 10); or L-NAME and compound 21 and olmesartan medoxomil (LN + Olm + C21; n = 10) for 6 weeks. L-NAME was administered in drinking water, whereas compound 21, olmesartan, and their combination were applied orally by pipette once daily between 8:30 AM and 10:00 AM. Control animals were sham-pipetted with an equal volume of vehicle (water for injection). Animals were housed under standard laboratory conditions (temperature: 23 ± 1°C; 12-hour light-dark cycle), and they were fed a standard pellet diet (1% NaCl) and drank tap water ad libitum. All of the procedures and experimental protocols, which were approved by the Landesamt fuer Gesundheit und Soziales (Berlin, Germany), conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

#### Noninvasive BP Measurement

Systolic BP was measured each week by tail-cuff plethysmography (ADInstruments, Spechbach, Germany), following a 2-week pre-treatment training and accommodation period.

#### Invasive BP and PWV Measurement

The rats were placed on a heated (37°C) plate under 1.5% isoflurane inhalation anesthesia. Two microtip catheters (Samba Sensors AB, Västra Frölunda, Sweden) were placed, one in the carotid and one in the femoral artery. The pressure data were displayed and recorded using an A/D converter with Chart version 6.0 Software (ADInstruments). The carotid catheter was placed in the aortic arch to the closest location to the aortic valve at which the valve was not interfering with the catheter measurement. The femoral catheter was then inserted until it reached the same location as the carotid catheter in the aortic arch (which was determined according to the pressure curve) and then retracted by 10 cm.

From the data recorded, heart rate, systolic BP, mean arterial pressure, diastolic BP, and pulse pressure were extracted using the built-in routines. The recorded pulse waves were manually analyzed (15 per animal) to determine the inflection point. Augmentation pressure was then calculated as the pressure from inflection point to systolic BP and augmentation index as augmentation pressure to pulse pressure percentage. The foot of the pulse waves was manually determined in both the carotid and femoral catheter (15 per animal). The duration from the foot of the wave recorded by the carotid catheter to the wave recorded by the femoral catheter (Δt) was used to calculate PWV = 10 cm/Δt and expressed as meters per second.

#### Aortic Morphometry and Elastic Modulus Calculation

Segments of thoracic aorta (from 5 to 10 mm above diaphragm) were fixed for 24 hours in 4% paraformaldehyde. Then they were embedded in paraffin, cut in serial 5-μm-thick sections, and stained with hematoxylin and eosin. The intima plus media wall thickness (WT; 8 per aorta) was determined using a ×40 objective, and the inner circumference (IC) was determined using a ×5 objective using the morphometric ImageJ version 1.33 software (National Institutes of Health). Finally, the inner diameter, ID = IC/π, and cross-sectional area, CSA = π ⋅ [(ID/2 + WT)² − (ID/2)²] were calculated.

Because of logistic reasons (preapproved number of animals that needed to be used for both biochemical and histomorphological analyses), the aortas were not perfused at the time of isolation. This provides a certain limitation of our method because the vessels could collapse. Therefore, the values for ID and the derived calculations should be considered with caution. However, all of the aortas were handled the same way, and the observed differences between the groups should point to the same direction irrespective of whether perfusion was used. Moreover, to minimize the error caused by vessel collapse, only well-preserved and round-shaped arteries were evaluated (n = 15 for Ctrl, 13 for L-NAME, 14 for LN + C21, 10 for LN + Olm, and 10 for LN + Olm + C21). The elastic modulus was calculated from the Moens-Korteweg equation, $E = PWV^2/\rho/WT$, where $\rho$ was estimated as 1060 kg/m³, with the limitation that the estimated value of the elastic modulus relates to given pressure, WT, and ID only.

#### Determination of Hydroxyproline Concentration

Samples from the thoracic aorta (8 mg) were dried at 100°C for 24 hours. The tissue was then hydrolyzed with 100 μL of 4-mol/L NaOH at 120°C for 10 minutes. After neutralization with 100 μL of 1.4-mol/L citrate, oxidation of the hydrolyte (10 μL) was initiated with the addition of chloramine-T reagent (1.0 mL) at 25°C. The reaction was stopped after 20 minutes by adding Ehrlich aldehyde reagent (1.0 mL). After incubation of samples at 65°C for 15 minutes, the absorbance of developed chromophore at 550 nm was measured. Concentration of hydroxyproline was determined from the standard curve and expressed as micrograms per milligram of sample dry weight.

#### Statistics

Data are presented as mean±SEM. Results were considered significant if $P < 0.05$. One-way, 2-tailed ANOVA with Bonferroni post-test for unpaired values and repeated-measures ANOVA for paired values was used. Normality was tested according to Kolmogorov and Smirnoff, and the difference in SDs was tested by the Barlett test.
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Results

Blood Pressure

l-NAME administration caused a progressive increase in systolic BP from 108.5±3.4 mm Hg before treatment to 170.6±8.6 mm Hg after 6 weeks of treatment and was higher from week 1 of treatment compared with the control group (P<0.05 versus Ctrl) in which systolic BP rose slightly from 109.3±2.0 to 117.7±2.9 mm Hg.

In the LN+C21 group, systolic BP rose from 111.7±3.7 to 156.5±5.4 mm Hg and was higher compared with the control group from week 1 of treatment (P<0.05 versus Ctrl) but was not significantly different from the l-NAME–treated group. The systolic BP in the LN+Olm group rose from 111.3±1.8 to 119.0±5.0 mm Hg and was not significantly different from control values, and from week 2 of treatment it was lower compared with rats treated with l-NAME alone (P<0.05 versus l-NAME; Figure 1A).

Body Weight and Organ Weights

At the end of the experiment, the body weight in the control group was 459.3±10.3 g, and the tibia length was 4.09±0.06 cm. None of the treatments significantly altered the body weight or tibia length. The left ventricular weight at the end of the experiment was 889.5±25.7 mg, and its ratio to tibia length was 219.7±3.3 mg/cm. l-NAME administration increased left ventricular weight by 14.4% (P<0.05 versus Ctrl) and the ratio by 13.2% (P<0.05). Although treatment with compound 21 did not prevent absolute or relative left ventricular hypertrophy, both regimens containing olmesartan completely prevented left ventricular hypertrophy development (Table 1).

In Vivo Catheterization and PWV Measurement

At the time point of PWV measurement, the heart rate in the control group was 322±9.6 bpm, and there was no difference in heart rate between the groups investigated. The mean arterial pressure was 91.4±3.6 mm Hg in the control group, whereas it was 121.9±9.1 mm Hg in the l-NAME–treated group. The mean arterial pressure did not differ in the LN+C21 group from rats treated with l-NAME alone, whereas in rats treated with olmesartan (with or without compound 21), it was not different from controls.

Augmentation index in the control group was 16.1±2.2%. In l-NAME–treated rats, the augmentation index was increased to 23.2±4.5% (P<0.05 versus Ctrl). Treatment of l-NAME rats with compound 21 partly prevented augmen-

Table 1. Basic Parameters After 6 Wk of Treatment

<table>
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<tr>
<th>Parameter</th>
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<th>LN+Olm</th>
<th>LN+Olm+C21</th>
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<td>BW, g</td>
<td>459±10</td>
<td>475±0.3</td>
<td>466±8</td>
<td>458±8</td>
<td>452±10</td>
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<td>Tibia, cm</td>
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<td>4.11±0.03</td>
<td>4.11±0.04</td>
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<td>LVW, mg</td>
<td>890±26</td>
<td>1018±31*</td>
<td>993±33*</td>
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<td>LVW/T, mg/cm</td>
<td>220±3.2</td>
<td>249±9.2*</td>
<td>245±8.6*</td>
<td>201.2±3.7†</td>
<td>203±6.1†</td>
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<td>ID, mm</td>
<td>1.79±0.06</td>
<td>1.56±0.04*</td>
<td>1.59±0.03*</td>
<td>1.48±0.03*</td>
<td>1.54±0.04*</td>
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<td>CSA, mm$^2$</td>
<td>0.40±0.04</td>
<td>0.43±0.03</td>
<td>0.40±0.02</td>
<td>0.34±0.3</td>
<td>0.35±0.04</td>
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</table>

Values are mean±SEM. Ctrl indicates control after 6 wk of experiment; l-NAME, 50 mg/kg per d of N$^\text{G}$-nitro-L-arginine methyl ester; LN+C21, l-NAME+0.3 mg/kg per d of compound 21; LN+Olm, l-NAME+10 mg/kg per d of olmesartan medoxomil; LN+Olm+C21, l-NAME+olmesartan medoxomil+compound 21; BW, body weight; LVW, left ventricular weight; ID, aortic inner diameter; CSA, aortic cross-sectional area. Only the following number of of animals per group was suitable was for morphometric evaluation (ID, CSA): 15 (Ctrl), 13 (l-NAME), 14 (LN+C21), 10 (LN+Olm), and 10 (LN+Olm+C21).

*P<0.05 vs Ctrl by 2-tailed, 1-way ANOVA Bonferroni.

†P<0.05 vs l-NAME by 2-tailed, 1-way ANOVA Bonferroni.
tation index increase to 21.0±2.4% (not significant [NS] versus Ctrl, NS versus l-NAME). Olmesartan and olmesartan+compound 21 treatment limited augmentation index increase to 17.1±3.2% and 16.0±3.3%, respectively (NS versus Ctrl, P<0.05 versus l-NAME, both; Table 2).

PWV in the control group was 3.25±0.09 m/s. In l-NAME-treated rats, the PWV was increased to 4.8±0.37 m/s (P<0.05 versus Ctrl). Treatment of l-NAME rats with compound 21 partly prevented PWV increase to 4.01±0.18 m/s (P<0.05 versus Ctrl, P<0.05 versus l-NAME). Olmesartan and olmesartan+compound 21 treatment prevented PWV increase to 3.43±0.21 m/s and 3.11±0.15 (NS versus Ctrl, P<0.05 versus l-NAME, both), respectively (Figure 1B).

Arterial Remodeling and Elastic Modulus Calculation
In the control group, the aortic WT was 129±8.5 µm, ID was 1789±61 µm, the WT/ID ratio was 72±3.1 µm/mm, and the cross-sectional area was 0.40±0.04 mm². l-NAME administration led to arterial remodeling characterized by increased WT (157±11.9 µm; P<0.05 versus Ctrl), reduced ID (1562±43 µm; P<0.05 versus Ctrl), increased WT/ID ratio (102±9.8 µm/mm; P<0.05 versus Ctrl), and only slightly increased CSA (0.43±0.03 mm²; NS versus Ctrl). Compound 21 partly prevented wall thickening (142±3.2 µm; P<0.05 versus Ctrl) without affecting ID or CSA, leading to reduced WT/ID ratio (91±2.1 µm/mm; P<0.05 versus Ctrl, P<0.05 versus l-NAME). Treatments with olmesartan and olmesartan+compound 21 prevented wall thickening (134±4.5 µm and 132±4.2 µm, respectively, NS versus Ctrl, P<0.05 versus l-NAME for both) without affecting ID and slightly reducing CSA. Treatment with olmesartan alone partly (91±4.6 µm/mm, P<0.05 versus Ctrl, P<0.05 versus l-NAME) and in combination with compound 21 completely prevented the increase of the WT/ID (86±5.2 µm/mm, NS versus Ctrl, P<0.05 versus l-NAME; Table 1 and Figures 2 and 3).

The elastic modulus in the aorta in the control group was 157±10 kg/m per second squared. In l-NAME-treated rats, the elastic modulus was increased to 247±12 kg/m per second squared (P<0.05 versus Ctrl). Treatment of l-NAME rats with compound 21 prevented the increase in elastic modulus to 189±15 kg/m per second squared (P<0.05 versus Ctrl, P<0.05 versus l-NAME). Olmesartan treatment prevented elastic modulus augmentation to 137±13 kg/m per second squared (NS versus Ctrl, P<0.05 versus l-NAME), and olmesartan+compound 21 reduced the elastic modulus below the control values to 120±12 kg/m per second squared (P<0.05 versus Ctrl, P<0.05 versus l-NAME; Figure 4A).

<table>
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<tr>
<th>Parameter</th>
<th>Ctrl</th>
<th>l-NAME</th>
<th>LN+C21</th>
<th>LN+Olm</th>
<th>LN+Olm+C21</th>
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<tr>
<td>HR, bpm</td>
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<td>317±32</td>
<td>324±12</td>
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<td>MAP, mm Hg</td>
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<td>122±9.1*</td>
<td>126±10.3*</td>
<td>92±8.5†</td>
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<td>SBP, mm Hg</td>
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<td>141±8.8*</td>
<td>145±11.8*</td>
<td>110±4.2†</td>
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<td>DBP, mm Hg</td>
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<td>105±2.4*</td>
<td>108±9.7*</td>
<td>75±2.5†</td>
<td>73±2.1†</td>
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<td>PP, mm Hg</td>
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<td>36±2.3*</td>
<td>35±1.9†</td>
<td>36±1.8‡</td>
</tr>
<tr>
<td>AP, mm Hg</td>
<td>5.7±0.9</td>
<td>8.2±1.6*</td>
<td>7.9±1.2</td>
<td>5.8±1.1†</td>
<td>5.6±0.9‡</td>
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<td>Aix, %</td>
<td>16±2.2</td>
<td>23±4.5*</td>
<td>20.1±2.4*</td>
<td>17±3.2†</td>
<td>16±3.2†</td>
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Values are mean±SEM. Ctrl indicates control after 6 wk of experiment; l-NAME, 50 mg/kg per d of N^ω-nitro-arginine methyl ester; LN+C21, l-NAME+0.3 mg/kg per d of compound 21; LN+Olm, l-NAME+10 mg/kg per d of olmesartan medoxomil; LN+Olm+C21, l-NAME+olmesartan medoxomil+compound 21; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; AP, augmentation pressure; Aix, augmentation index.

*P<0.05 vs Ctrl by 2-tailed, 1-way ANOVA Bonferroni.
†P<0.05 vs l-NAME by 2-tailed, 1-way ANOVA Bonferroni.

Figure 2. Aortic wall thickness (A) and aortic wall thickness to inner diameter ratio (B) in N^ω-nitro-arginine-methyl ester (l-NAME; 50 mg/kg per d for 6 wk)-treated rats. Only well-preserved, round-shaped structures were evaluated (n=15 for vehicle [Ctrl], 13 for l-NAME, 14 for LN+C21, 10 for LN+Olm, and 10 for LN+Olm+C21). *P<0.05 vs Ctrl, †P<0.05 vs l-NAME. Values are mean±SEM; P, ANOVA Bonferroni.
Aortic Hydroxyproline

The hydroxyproline concentration in the aorta in the control group was 31.2±0.9 µg/mg. In L-NAME–treated rats, the hydroxyproline concentration was increased to 39.7±2.6 µg/mg (P<0.05 versus Ctrl). Treatment of L-NAME rats with compound 21 prevented the increase in hydroxyproline concentration to 33.8±1.9 µg/mg (P<0.05 versus Ctrl, L-NAME). Olmesartan treatment prevented hydroxyproline accumulation to 35.9±2.5 µg/mg (0.05 versus Ctrl, P<0.05 versus L-NAME), and olmesartan+compound 21 completely prevented hydroxyproline accumulation to 29.9±1.5 µg/mg (NS versus Ctrl, P<0.05 versus L-NAME, P<0.05 versus LN+OlE; Figure 4B).

Discussion

In the present study we investigated the effects of direct AT2R stimulation on PWV and aortic remodeling in L-NAME–treated rats. L-NAME administration caused a progressive increase in BP accompanied by increased PWV. These changes were associated with thickening of the aortic wall, increased elastic modulus, and collagen accumulation in the aorta. The AT1R blockade completely prevented hypertension, PWV, aortic WT, and elastic modulus increase and partly prevented hydroxyproline accumulation in the aorta. Selective AT2R stimulation partly prevented all of these alterations, yet without concomitant prevention of BP rise. Combination therapy with olmesartan medoxomil and compound 21 led to BP levels, PWV, and WT comparable to olmesartan-alone–treated rats, but only in the combination group complete prevention of increased hydroxyproline deposition was achieved, resulting in even more pronounced stiffness reduction. Notably, all AT2R agonist–induced alterations in arterial stiffness and wave velocity were independent from any effect on BP.

In our current study, L-NAME administration caused BP elevation, which was associated with increased augmentation index and PWV. The effect of L-NAME administration on BP was reported in numerous studies by our laboratory \cite{31,32} and others. L-NAME–induced hypertension is associated with target organ damage, such as cardiac hypertrophy and fibrosis, vascular remodeling, and endothelial damage. Current European guidelines also regard augmented PWV as an indicator of subclinical organ damage. PWV is considered the best and direct marker of arterial stiffness. Both experimental and clinical hypertension \cite{34,35} are associated with increased PWV. The PWV is considered to be an independent cardiovascular risk factor not only in hypertensives but also in end-stage renal disease patients, in the elderly, and even in the general population. In a recently published meta-analysis, increased arterial stiffness was linked to a 2-fold increase in cardiovascular events and mortality, as well as all-cause mortality.

Aortic stiffening partly depends on actual BP levels and might be acutely reversed by BP lowering. However, when structural vascular alterations are present, the aortic stiffness is less responsive to BP reduction. Moreover, the failure to reduce aortic stiffness in response to BP lowering...
seems to have a negative impact on the survival of patients with end-stage renal failure patients. Therefore, there is an unmet medical need for novel agents with destiffening properties without or beyond BP-lowering effects.

In addition to being determined by BP, PWV relates to endothelial function and NO insufficiency or the long-term effect of the hemodynamic overload on arterial wall remodeling. In fact, PWV is influenced by the vessel wall geometry reflected by WT/ID ratio and by the elastic modulus that reflects the changes in the elastic properties of the arterial wall itself. In the present study we observed that aortic remodeling in l-NAME hypertension was characterized by increased aortic WT similarly to our previous studies. The wall thickening was accompanied by reduced ID, slightly increased aortic cross-sectional area, and most importantly increased WT/ID ratio in accordance with previous studies on this model of hypertension.

In our study we have investigated the effects of the AT2R agonist on PWV in l-NAME rats. Direct AT2R stimulation was already reported to reduce the extent of myocardial infarction and to improve cardiac function along with anti-inflammatory effects in rats. The effects of AT2R receptor stimulation across a broad field of experimental models seem to be BP neutral, which was also confirmed in our model of l-NAME hypertension, where C21 did not significantly reduce BP, neither alone nor as an additive effect when added on top of olmesartan treatment. The beneficial effects of AT2R stimulation seem to be related to its anti-inflammatory effects that, in turn, are believed to be mediated by inhibition of nuclear factor-kB activity and translocation, by the activation of intracellular protein tyrosine phosphatase SH2 (src homology 2) domain-containing phosphatase 1, and by the inhibition of cyclooxygenase activity. Inflammation not only plays an important role in the pathogenesis of hypertension and the development of target organ damage, but acute systemic inflammation was also shown to increase arterial stiffness in healthy individuals as well. We, therefore, hypothesized that the stimulation of the AT2R might exert beneficial effects on aortic stiffness. In our experiment, we observed that treatment with compound 21 partly prevented the increase in PWV in l-NAME rats along with prevention of aortic wall thickening even without any significant prevention of BP increase. We have compared the effects of compound 21 to olmesartan at a high 10-mg/kg antihypertensive dose that completely prevented hypertension. AT2R antagonists currently not only belong to the gold standard therapies for hypertension and cardioprotection at various stages of the cardiovascular continuum but have shown the highest potential for preventing and reducing PWV in hypertension. Also in our study olmesartan completely prevented PWV increase and aortic wall thickening. However, the effects on PWV might depend on the actual BP level and aortic geometry. Therefore, we have estimated the elastic modulus of the aorta in the groups investigated. The elastic modulus increase was not only completely prevented by olmesartan but even more influenced by the combined treatment with olmesartan and compound 21, without additional BP lowering by the AT1R agonist. The fact that the vessel dimensions were not investigated in perfused aortas provides a certain limitation to our data on the elastic modulus. However, we have investigated other parameters that could indicate altered elastic properties of the vascular wall independent of histological evaluation to assess whether these are in line with the favorable effects on vascular structure suggested by the elastic modulus data.

Of the aortic wall components, collagen shows the highest elastic modulus and, therefore, appears to be the major determinant of aortic stiffness. In our study we have determined the level of hydroxyproline, a marker of collagen, in the aorta. Although both compound 21 and olmesartan partly prevented the excessive collagen deposition in the aorta, their combination completely prevented the accumulation of collagen even when compared with treatment with olmesartan alone.

Some of the AT2R-mediated effects are attributed to enhancement of NO release. NO represents a molecule with antiproliferative and antiproteosynthetic properties. However, in our experiment compound 21 was given together with l-NAME. The inhibition of NO synthase by L-NAME is difficult to counterbalance, and the effects observed in our study are most likely NO independent and might relate to the direct antiproteosynthetic and antiproliferative properties of AT2R per se. Most pathological parameters in our study were only partly restored by AT2R stimulation, suggesting that effects coupled to the AT2R are partly NO dependent and partly NO independent. On the other hand, incomplete restoration could also be attributed to the dose of compound 21 used. It might be regarded as a limitation of this study that no higher dose than 0.3 mg/kg was tested. However, in 2 previous studies, a dose of 0.3 mg/kg of compound 21 did not result in any further outcome improvement when compared with 0.03 mg/kg. Thus, we supposed the 0.3-mg/kg dose to be appropriate for sufficient AT2R stimulation in our in vivo study.

We conclude that a chronic treatment with the direct AT2R agonist compound 21 partly prevented aortic PWV increase, wall thickening, and elastic modulus augmentation, along with reduced collagen deposition, without preventing hypertension in rats with NO synthase inhibited by l-NAME. The effects on elastic modulus and collagen accumulation were additive to AT2R blocker action, yet without additional BP-lowering effect. Therefore, these effects of compound 21 treatment seem to be NO and BP independent.

**Perspectives**

PWV is an established cardiovascular risk factor associated with hypertension. However, BP lowering does not always lead to PWV reduction, and after BP reduction without PWV normalization the cardiovascular risk remains elevated. Therefore, there is an unrelenting search for novel agents with destiffening properties without or beyond BP-lowering effects. Our study demonstrates that direct AT2R stimulation provides means for BP-independent prevention of PWV increase that might be additive to the antihypertensive effects of an AT1R blockade. The search for mechanisms underlying these effects warrants further research.
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Disclosures
B.D. and T.U. received speaker fees from Vicore Pharma. T.U. has a modest interest in Vicore Pharma. B.D. has a significant interest in Mintage Scientific, the owner of Vicore Pharma.

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