The Effect of a Nonpeptide Angiotensin II Type 2 Receptor Agonist, Compound 21, on Aortic Aneurysm Growth in a Mouse Model of Marfan Syndrome

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INTRODUCTION

Marfan syndrome (MFS) is a multisystem connective tissue disorder characterized by cardiovascular, musculoskeletal, and ocular abnormalities. Most life-threatening for these patients are thoracic aortic aneurysm (TAA) and dissection. In the past decades, life expectancy of Marfan patients increased due to earlier diagnosis and proper surgical management of aortic disease. The optimal medical therapy for these patients is still not clear. Nowadays, treatment focuses mainly on interfering with hemodynamics, using beta-blockers.

Marfan patients have a defect in fibrillin-1. This is associated with disturbances in transforming growth factor-beta (TGF-β) and angiotensin II (Ang II) signaling. These processes are accompanied by fragmentation of elastin, excess of metalloproteinases, and infiltration of macrophages, causing an inappropriate remodeling within the extracellular matrix and aortic root dilatation. Evidence suggests that Ang II signaling might be a good target for medical intervention. In murine models of MFS, the angiotensin II type 1 receptor (AT1R) antagonist losartan has been shown to effectively slow down aortic growth in comparison with enalapril (ACE-I). The net effect of an AT1R antagonist is not clear because these agents increase not only Ang II but also angiotensin II type 2 receptor (AT2R) expression. AT2R is known to exert antiproliferative, anti-inflammatory, and cardioprotective effects that partly counterbalance the effects of AT1R stimulation. Moreover, the protective effect of AT2R blocker losartan was lost in fibrillin-1-deficient mice with disruption of AT2R gene. Therefore, it is believed that losartan shunts Ang II signaling through the AT2R, which seems to have a protective role in the development of TAA.

Because C21 is the first highly selective nonpeptide AT2R agonist, it might be a good therapeutic agent for the prevention of aortic aneurysm growth in MFS. The compound previously showed to have several beneficial cardiovascular effects. It reduces myocardial fibrosis and vascular injury, promotes vasodilatation, reduces infarct size, and delays the development of aortic atherosclerosis. The primary aim of this study was to investigate whether C21 decreases aneurysm growth in a mouse model of the MFS.

METHODS

Murine Model

Approval for the project was obtained from the Ethical Commission at the KULeuven (number: P060/2012).

Male mice heterozygous for a cysteine substitution in an epidermal growth factor–like domain of fibrillin-1 (Fbn1C1039G+) were used. These mice develop...
pathological changes in the aortic wall and aneurysms of the ascending aorta. Wild-type (WT) littermates (C56BL/6J) were used as controls. At an age of 8 weeks, mice were randomly assigned to 1 of the 8 groups (n = 14 per group) (Figure 1). Thereafter, treatment was started and continued for 6 months. In case of early mortality, an autopsy excluded aortic causes of death. Intraperitoneal (IP) injection [C21 vs. NaCl (0.9%)] was performed daily. Other drugs (losartan and enalapril) were administered through drinking water, in doses which were used in previous studies.\(^{12}\) Originally, these doses were chosen because they achieve a comparable hemodynamic effect.\(^{20}\) Echographic evaluation and blood pressure measurements were performed at 0, 3, and 6 months of treatment. Thereafter, an overdose of pentobarbital killed the animals. Immediately following sacrifice, the right atrial appendix was opened. Through a puncture of the left ventricle, the aorta was imaged in the parasternal long axis view. Three separate measurements of the maximal internal diameter at the level of the sinuses of Valsalva during systole were obtained by an observer blinded to genotype and treatment arm (JS).

**Echographic Evaluation**

The aortic root of the mice was imaged using a high-resolution transthoracic echography (Vevo 770; VisualSonics, Toronto, Canada), and a 40-MHz transducer. Nair hair removal cream was used at least 1 hour before echocardiography. The mice were sedated with isoflurane inhalation. The aorta was imaged in the parasternal long axis view. Three separate measurements of the maximal internal diameter at the level of the sinuses of Valsalva during systole were obtained by an observer blinded to genotype and treatment arm (JS).

**Histology**

Serial cross-sections (7 \(\mu\)m, 10 series) were made after paraffin embedding (Microm HM360; Thermo Scientific, Walldorf, Germany). Hematoxylin and eosin as well as Verhoeff–Van Gieson (VVG) staining were performed. Images of the ascending aorta were obtained at the level of the sinotubular junction at \(\times40\) magnification using a Zeiss Axiosplan microscope and an Axiocam MRc5 camera. Two blinded observers (D.V. and M.C.) performed measurements using ImageJ software and results were averaged. Media thickness, the average of 8 measurements (2 per quadrant), was obtained using hematoxylin and eosin–stained samples. VVG was used to evaluate wall architecture at 4 different locations, using an arbitrary scale based on the number of elastin breaks per cross-section. A “break” was defined as an isolated area where an elastic fiber was fragmented with intersected excessive connective tissue matrix. A scale of (1) indicated no breaks, (2) 1 to 2 breaks, (3) 3 to 4 breaks, (4) 5 to 6 breaks, and (5) 7 or more breaks.\(^{12}\) Dividing the area of elastic fibers by the total area of the media determined the elastin fiber content.\(^{22}\)

**Plasma Concentration**

Eight-week-old WT mice were administered NaCl (0.9%), normal dose C21 (0.5 mg \(\text{kg}^{-1}\cdot\text{d}^{-1}\)), or high dose

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**FIGURE 1.** Protocol showing timing, initiation, and duration of therapy for the different groups, based on genotype and treatment. This for the long-term study (A) and the early-start study (B).
C21 (5.0 mg·kg⁻¹·d⁻¹) for 7 consecutive days intraperitoneally. Thereafter, the animals were euthanized 1, 6, 12, and 24 hours after the past injections (n = 3 per time and dose). Blood was sampled from the right atrium using ethylenediaminetetraacetic acid solution. The blood was centrifuged (2500 g for 10 minutes). Plasma was stored at −80°C. Determination of the plasma concentration was provided by Vicore Pharma (Data on file; Vicore Pharma, Mölndal, Sweden). In general, C21 was extracted from mouse plasma by addition of acetonitrile and determined using ultra performance liquid chromatography. Based on the exponential best-fit line, plasma half-time was calculated.

**Statistics**

Statistical analysis was conducted using SPSS (IBM SPSS statistics; IBM Corporation, Armonk, NY). The Kolmogorov–Smirnov test evaluated normal distribution and the Levene’s test evaluated the homogeneity of variance. One-way analysis of variance (ANOVA) with post hoc Scheffe’s correction was used to compare mean values. A $P$-value of ≤0.05 was set for
statistical significance. Data are presented as mean ± 2 SEM. Measurements were not corrected for weight of the mice because there were no differences between the groups after 3 and 6 months of treatment.

RESULTS
Murine Model
Ten mice (9%) died before the end of the experiment. Two in the WT group, the others were Fbn1C1039G/+ distributed over different treatment groups (no significant difference). Autopsy revealed a retroperitoneal hematoma in one of the losartan-treated animals, but without dissection or dilatation of the thoracic aorta. In all other animals, there were no signs of aortic dissection or rupture. Figure 2 shows macroscopic images of the heart and ascending aorta of the different groups.

Blood Pressure
Diastolic and systolic blood pressures at baseline, after 3 months, and 6 months of treatment were comparable in all groups. Figure 3 shows the diastolic and systolic blood pressures at the end of the experiment.

Echographic Evaluation
There was a significant increase in aortic sinus diameter at 6 months in all groups except in WT and losartan-treated mice (Table 1). In the C21 0.5 mg/kg treated mice, the sinuses of Valsalva were already significantly larger after 3 months of treatment.

At the age of 8 weeks, the aortic root in mutant mice is larger than in WT mice: 1.69 ± 0.09 mm versus 1.909 ± 0.032, respectively (P < 0.001). After 3 months, the diameter in vehicle and C21 0.5 mg/kg treated Fbn1C1039G/+ mice was already significantly larger than in WT mice (Figure 4). Another 3 months later, the diameter of the C21 5 mg/kg, enalapril, and enalapril-C21–treated mice was also significantly larger. At this point in time, there was no difference between WT and Fbn1C1039G/+ mice treated with losartan, with or without C21 associated.

| TABLE 1. Mean Aortic Sinus Diameter, 2 SEM, Number of Measured Animals per Group, and P-value Compared With the Start of the Experiment |
|-----------------|----------------|----------------|------------------|----------------|----------------|----------------|
| Group           | Month 0        | Month 3        | Month 6         |
|                 | Mean ± 2 SEM  | n              | Mean ± 2 SEM  | n              | Mean ± 2 SEM  | n              |
| (A) WT          | 1.68 ± 0.08   | 12             | 1.76 ± 0.06   | 12             | 1.73 ± 0.08   | 12             |
| (B) Vehicle     | 1.84 ± 0.12   | 11             | 2.17 ± 0.14   | 11             | 2.27 ± 0.28   | 11             |
| (C) C21 0.5 mg/kg | 1.83 ± 0.09 | 14             | 2.17 ± 0.18   | 14             | 2.19 ± 0.16   | 14             |
| (D) C21 5 mg/kg | 1.77 ± 0.08   | 13             | 1.92 ± 0.18   | 12             | 2.20 ± 0.09   | 10             |
| (E) Losartan    | 1.85 ± 0.09   | 13             | 1.85 ± 0.11   | 11             | 1.99 ± 0.10   | 10             |
| (F) Losartan—C21 0.5 mg/kg | 1.86 ± 0.15 | 13             | 1.93 ± 0.10   | 12             | 2.09 ± 0.13   | 12             |
| (G) Enalapril   | 1.90 ± 0.06   | 14             | 1.89 ± 0.12   | 14             | 2.23 ± 0.14   | 12             |
| (H) Enalapril—C21 0.5 mg/kg | 1.95 ± 0.05 | 14             | 1.99 ± 0.11   | 14             | 2.18 ± 0.18   | 11             |

FIGURE 4. Average aortic root diameter (±2 SEM) at start, after 3 months, and 6 months of treatment. Number of animals at the top of each column. Horizontal line indicates significance (P-value at the end of each line).
Histology
A representative sample of VVG staining for each group is depicted in Figure 5. The media of the WT, losartan, and enalapril-treated mice were thinner although not significantly (Table 2). Quantification of the elastin fragmentation could not reveal any difference between the groups, nor the elastin fiber content.

Plasma Concentration
The plasma concentration of C21 in the control group (NaCl 0.9%) was 0 ng/mL. The plasma concentrations after injection of C21 is depicted in Figure 6. The plasma half-time is 2.5 and 2.2 hours for 0.5 and 5.0 mg $\cdot$ kg$^{-1}$ $\cdot$ d$^{-1}$, respectively.

Early Start
At the age of 8 weeks, the echographic diameter of the aortic sinuses was significantly larger in the Fbn1C1039G/+ mice independent of treatment (Table 3). Histological evaluation showed a significant increase in media thickness and elastin breaks in the mutated mice. The elastic fiber content was comparable in all groups.

DISCUSSION
It is believed that AT1R is primarily involved in pathophysiological actions, whereas AT2R signaling antagonizes many of the effects of AT1R signaling. Studies suggest that sartan-mediated effects occur at least in part because of the unopposed action of the AT2R. This highlights the potential of the AT2R as a therapeutic target for blunting aortic aneurysm formation, the key idea of this study.

Serial blood pressure measurements were performed because blood pressure lowering has a beneficial effect on aortic aneurysm growth. There were no significant differences between the treatment groups in systolic, diastolic, or mean blood pressure at the start nor after 3 and 6 months of treatment. The administered doses of losartan and enalapril have a comparable blood pressure lowering effect, as described previously. In this study, lower systolic and diastolic blood pressures, although not statistically significant, occurred in those 2 groups. Data on hemodynamic effects of C21 are controversial. Most of the ex vivo studies report vasodilatation and thus potentially a fall in blood pressure. By contrast, as in this study, C21 did not decrease blood pressure in vivo. Evaluation of the growth of the aortic root showed a significant increase in diameter in all groups except in the WT and losartan-treated animals, independently of C21 association. Comparing echographic measurements between groups at the age of 8 months confirmed that treatment with losartan was the only effective one. Evaluation of media thickness, architecture score, and elastic fiber content could not reveal any difference between groups. The reason for the lack of histological effect of any treatment, even losartan, is unclear. One of the principal findings of Habashi et al was that losartan achieved full correction of the phenotypic abnormalities in the aortic wall of Fbn1C1039G/+ mice. The age at the start, duration of the treatment, and sample size were comparable. In this study, cross-sections were taken at the level of the sinotubular junction and not the aortic root, this might attribute to differences in histological results. Another reason might be the difference in losartan intake. The objective was to have an intake of 50

| Group          | Wall Thickness (µm) | Elastin Break | Elastin Fiber Content (%) |
|----------------|---------------------|---------------|---------------------------|
|                | Mean 2 SEM n        | Mean 2 SEM n  | Mean 2 SEM n              |
| WT             | 55.51 6.91 8        | 1.2 0.1 8    | 54.09 4.06 8              |
| Vehicle        | 71.03 13.85 8      | 1.8 0.3 8    | 43.94 3.17 8              |
| C21 0.5 mg/kg  | 80.41 8.16 10      | 1.9 0.4 12   | 49.49 5.59 12             |
| C21 5 mg/kg    | 67.15 16.85 7      | 1.8 0.5 8    | 51.42 7.59 8              |
| Losartan       | 57.18 7.61 7       | 1.9 0.3 8    | 45.66 4.47 8              |
| Losartan—C21 0.5 mg/kg | 78.07 20.84 5     | 1.9 0.9 7    | 53.80 6.50 7              |
| Enalapril      | 58.99 15.06 8      | 2.0 0.5 8    | 48.10 4.54 8              |
| Enalapril—C21 0.5 mg/kg | 70.55 14.74 9     | 2.2 0.6 9    | 47.48 8.04 9              |
| $P$            | 0.056 0.177 0.206  |               |                           |
Based on the used losartan concentration in the drinking water of 250 mg/L, average monitored water intake per animal of 5.2 mL/d, and average mice weight throughout the experiment of 26.8 g, there was an average losartan intake of 49 mg·kg⁻¹·d⁻¹. In the described studies, the losartan concentration in the drinking water was more than double, at 600 mg/L. This means that the water intake of these mice was much lower, or that the real losartan intake was higher than the targeted 50 mg·kg⁻¹·d⁻¹. Higher losartan intake might have caused beneficial histological changes and lower blood pressures; 2 things which could not be validated in this study.

There are several possible reasons for the lack of benefit of C21 on aneurysm growth in this study. The first two which are discussed below, the timing of initiation of therapy and the doses used, are also the main limitations of this study. First, the treatment with C21, started at the age of 8 weeks, might be too late in the pathogenesis of aortic root dilatation in MFS. This timing was based on previous studies evaluating drug treatment in this mice model. However, the aortic root in Fbn1C1039G/+ mice undergoes progressive dilatation, evident as early as 2 weeks of age. In this study, at the age of 8 weeks, before the start of any treatment, the diameter of the aortic root was significantly larger in mutant compared with WT mice. Comparable differences have been described by Habashi et al. To overcome this, C21 was started at the age of 10 days in a subgroup of mice. This early treatment could not show any beneficial effect. At the age of 8 weeks, Fbn1C1039G/+ mice had a significantly larger aortic root diameter in comparison with WT mice, independently of treatment. This was confirmed with histological evaluation, showing aberrant thickening of the aortic media as well as fragmentation and disarray of elastic fibers. In these young mice, only the lower dose (0.5 mg·kg⁻¹·d⁻¹) was tested because this is best within the range of used doses of C21 in other models. Despite the lack of effect of C21 even in these young mice, a potential benefit of AT₂R agonism cannot be ruled out. This because, second, the used doses and administration route of C21 may not be ideal. C21 has been applied IP or orally at doses ranging from 0.01 to 10.0 mg/kg per day in different species. Because very little is known about the pharmacodynamics and pharmacokinetics of the studied compound, 2 doses (0.5 and 5 mg·kg⁻¹·d⁻¹) and 1 IP injection per day were chosen. Plasma concentrations were determined at specific time intervals after IP injection. The average calculated plasma half-time was 2.4 hours, which is in the range of previously described results after oral or intravenous administration in rats: 3–6 hours, and 0.5–2.5 hours, respectively. Alternatively, the compound could have been administered orally, generating a more constant plasma concentration. Then, the oral bioavailability of 20%–30% should be considered. Contrary to Ang II, which binds with similar affinity to the AT₁R and AT₂R, C21 is a highly specific AT₂R agonist. The affinity of C21 for the AT₂R is 25,000-fold higher than for the AT₁R. However, increasing the dose will not always enhance beneficial effects because high doses might stimulate the AT₁R. Therefore, lower doses of C21 might have
been needed to selectively stimulate AT2R.\textsuperscript{14} Stimulation of the AT1R effect can potentially be blocked by combining C21 with losartan,\textsuperscript{33} although this created no additional benefit in this study. Also, Chow et al\textsuperscript{17} found that coadministration of C21 and candesartan had no additive effect. However, administration of enalapril reduces the availability of Ang II for the AT1R and AT2R. Theoretically, adding C21 to enalapril treatment would have a comparable effect, blocking AT1R and stimulation of AT2R signaling, as isolated losartan treatment. Nevertheless, the aortic root diameter after 6 months’ treatment in the C21-enalapril group was significantly larger in comparison with the WT group. In addition to the capability of C21 to activate AT1R, the drug has also proven to have effects in an angiotensin-receptor–independent manner, such as vasorelaxation due to calcium entry blockade\textsuperscript{33} and antagonism of the thromboxane receptor.\textsuperscript{36} Thus, the absence of a beneficial effect of C21 on aneurysm growth might be due to the used doses, the administration route or non-AT2R mediated effects. Third, the lack of effect of C21 might be related to the unclear contribution of the RAA system, and more specifically of AT1R and AT2R, to aortic aneurysm progression. Initial studies characterizing thoracic aortic enlargement in MFS identified excessive TGF-β signaling as a driver of aortic disease.\textsuperscript{6} Because the AT1R antagonist losartan had been shown to inhibit TGF-β signaling, it was used to blunt TGF-β hyperactivity in the medial layer of Fbn1\textsuperscript{C1039G/+} mice. In this way, losartan showed to be more effective in mice than β-blockers and as effective as TGF-β-Nab in preventing aortic root enlargement and pathologic changes. On the contrary, clinical trials failed to validate these animal data.\textsuperscript{37,38} This puts in question whether TGF-β activation is truly the primary driver of aortic disease and whether losartan effect is exclusively the result of TGF-β inhibition.\textsuperscript{4,39} Some suggest that disruption of mechanosensing through the elastin-contractile complex can lead to aberrant AT1R activity and that this is the mode of action of losartan.\textsuperscript{2,39} The pathophysiological role of the AT2R on aortic aneurysm growth is probably even more complex. The presumed protective effect is based on blockade of the AT1R rather than direct stimulation of AT2R.\textsuperscript{12} Moreover, there is accumulating evidence to suggest that the role of AT1R is more than just the opposed action of the AT2R.\textsuperscript{11,33,40} Furthermore, the expression of AT2R varies a lot under physiological and pathophysiological conditions.\textsuperscript{41} One example is that expression and activity of the AT2R is highly enhanced in female animals.\textsuperscript{31,42,43} This means that there is probably a sex-specific role for the AT2R and its interfering drugs. It should be stated that this study was exclusively performed in male animals, in accordance with most other studies describing the effect of C21.\textsuperscript{14,16,17,27,32,35,44} To make things even more complex, angiotensin receptors might interfere with one another, like dimerization, affecting the consecutive signal transduction.\textsuperscript{40,45} To elucidate the role of the AT2R in aneurysm formation in MFS, the creation of a double knockout mice model combining an MFS model with a model with aortic overexpression of the AT1R might be interesting. A disadvantage of the used Fbn1\textsuperscript{C1039G/+} model is that long-term experiments are needed. This mouse model produces equal amounts of normal and abnormal fibrillin-1 and replicates the less commonly observed form of mild MFS.\textsuperscript{33} It was reported that by 6 months of age, more than 90% of these mice developed TAA of variable diameters, but only 5% died of a ruptured aneurysm by 8 months of age.\textsuperscript{46} In this study, there was no evidence of aortic aneurysm related mortality. The retroperitoneal hematoma in one losartan treated mouse was not associated with aneurysm and might be related to a traumatic IP injection. The use of another mouse model, Fbn1\textsuperscript{mgR/mgR}, which replicated the more frequently diagnosed form of progressively severe MFS, might be more valuable.\textsuperscript{47} These mice only produce approximately 20% of normal fibrillin-1, and aortic rupture is a fully penetrant manifestation within the first year of life. This has the additional advantage of including mouse survival as a more informative clinical endpoint.\textsuperscript{4} Moreover, there is evidence that the protective effect of losartan is not as clear in Fbn1\textsuperscript{mgR/mgR} mice, compared with Fbn1\textsuperscript{C1039G/+} mice,\textsuperscript{48,49} which reflects the disappointing results of the clinical trials in MFS evaluating the effect of losartan.\textsuperscript{37,38}

This study reveals that C21, a selective nonpeptide AT1R agonist, is ineffective—at the doses studied—to attenuate aneurysm growth in an MFS mouse model. Simultaneously, it was confirmed that specific AT1R antagonism is more effective when it comes to aortic root dimensions than the dual AT1R/AT2R blockade. Further studies are warranted to elucidate the exact role of the RAA system, and more specifically the role of the AT2R in aneurysm formation in MFS.

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**TABLE 3.** Average Aortic Root Diameter, Wall Thickness, Architecture Score, and Elastic Fiber Content of Early Start Experiment

| Group | Average Aortic Root Diameter (mm) | Wall Thickness (μm) | Architecture score | Elastic Fiber content (%) |
|-------|----------------------------------|---------------------|--------------------|--------------------------|
| WT    | Mean 2 SEM n                      | Mean 2 SEM n        | Mean 2 SEM n       | Mean 2 SEM n             |
| Vehicle | 1.63 0.06 8                      | 50.2 8.3 7          | 1.25 0.25 7        | 59.9 7.4 7               |
| C21 0.5 mg/kg | 1.90 0.06 12 | 68.5 7.3 12 | 1.82 0.29 12 | 51.2 4.2 12 |
| P     | <0.001                           | 0.001               | 0.032              | 0.121                    |

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