Nephroprotection by antifibrotic and anti-inflammatory effects of the vasopeptidase inhibitor AVE7688

OLIVER GROSS, MARIE-LOUISE KOEPKE, BOGDAN BEIROWSKI, ECKHARD SCHULZE-LOHOFF, STEPHAN SEGERER, and MANFRED WEBER

Department of Internal Medicine I, Medical Faculty University of Cologne, Cologne General Hospital, Merheim Medical Center, Cologne, Germany; and Medizinische Poliklinik, Klinikum Innenstadt, University of Munich, Munich, Germany

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Background. Chronic renal disease substantially increases the risk of cardiovascular events and death. Vasopeptidase inhibitors are known to show a strong antihypertensive effect. In the present study, we investigated the nephroprotective potential of the vasopeptidase inhibitor AVE7688 beyond its antihypertensive effects in a mouse model of progressive renal fibrosis.

Methods. COL4A3+/− mice received 25 mg AVE7688 per kg body weight. Treatment was initiated in week 4 (early) and week 7 (late). Eight mice per group were sacrificed after 7.5 or 9.5 weeks, and serum levels of urea, systemic blood pressure, and proteinuria were measured. Renal tissue was investigated by routine histology, electron microscopy, immunohistochemistry, and Western blotting. Lifespan until death from renal fibrosis was monitored.

Results. Lifespan of treated mice increased by 143% (early therapy) and by 53% (late therapy) compared to untreated animals (172 ± 19 vs. 109 ± 15 vs. 71 ± 6 days, P < 0.01). Untreated COL4A3+/− mice did not develop severe hypertension (mean systolic blood pressure 116 ± 14 vs. 111 ± 9 mm Hg in wild-type mice), and both therapies mildly reduced systemic blood pressure (107 ± 13 and 105 ± 14 mm Hg, data not significant). AVE7688 decreased proteinuria from 12 ± 3 g/L in untreated mice to 2 ± 1 g/L (early) and to 4 ± 1 g/L (late therapy, P < 0.05), as well as serum-urea from 247 ± 27 to 57 ± 10 and to 105 ± 20 mmol/L (P < 0.05). Extent of fibrosis, inflammation, and profibrotic cytokines was reduced by AVE7688 therapy.

Conclusion. The results indicate a strong nephroprotective effect of the vasopeptidase inhibitor in this animal model of progressive renal fibrosis. Besides the antihypertensive action of AVE7688, its antifibrotic, anti-inflammatory, and antiproteinuric effects demonstrated in the present study may serve as an important therapeutic option for chronic inflammatory and fibrotic diseases in man.

Chronic renal disease substantially increases the risk of cardiovascular events and death [1]. The common end point of most chronic renal diseases, such as chronic glomerulonephritis and diabetic nephropathy, is renal fibrosis. Tissue damage in chronic glomerulonephritis, for example, is related to the individual immune response, leading to excessive inflammation, failure to activate regression and glomerular repair, and excessive fibrogenic activity. Therefore, therapy of chronic renal diseases needs to target acute and chronic inflammation, as well as progressive renal fibrosis. At present, standard therapeutic regimens include pharmacologic blockade of the renin-angiotensin-aldosterone system by either inhibition of angiotensin-converting enzyme (ACE) or blockade of the angiotensin II receptor. Both therapies have been shown to delay progression of chronic renal diseases such as diabetic nephropathy and chronic glomerulonephritis [2–4]. However, despite some success of these therapies, chronic progressive renal disease continues to present a therapeutic challenge because the prevalence of patients on chronic renal replacement therapy increases by 5% to 7% per year in the United States and Europe. The limitations of current therapies stimulate research in this field and the development of new treatment options [5]. One strategy that received considerable attention is the development of vasopeptidase inhibitors [6, 7]. These drugs simultaneously inhibit the activity of ACE and neutral endopeptidase (NEP). NEP is an endothelial cell surface zinc metallopeptidase. It is the major enzymatic pathway of degradation of natriuretic peptides, and a secondary enzymatic pathway for degradation of kinins [8]. Vasopeptidase inhibitors were developed in an effort to generate superior effects compared to pure ACE inhibitors; however, their therapeutic value in renal end-organ damage has not yet been fully explored [9].

The vasopeptidase inhibitor omapatrilat has shown potent organ protection in models of salt-induced hypertension [10, 11], and in a hypertensive, hypoinsulinemic nephropathy model [12]. However, the potential benefit of pharmacologic intervention by vasopeptidase inhibitors has not been fully explored.

Key words: renal hypertension, inflammation, fibrosis, collagen, Alport syndrome.
inhibition in chronic progressive fibrotic renal diseases irrespective from its antihypertensive effects has not yet been evaluated.

In the present study, we investigated whether preemptive or late treatment with the novel vasopeptidase inhibitor AVE7688 has nephroprotective antifibrotic or anti-inflammatory effects in COL4A3 −/− mice. The COL4A3 −/− mice are a nonhypertensive animal model of progressive renal fibrosis [13–16], similar to the human Alport syndrome (AS) [13, 14]. AS is a hereditary nephropathy characterized by progressive renal failure, sensorineural deafness, and typical ocular changes [17]. The disease in mice and humans is caused by mutations in type IV collagen genes, leading to an abnormal composition of the glomerular basement membrane (GBM). Previous studies demonstrated that abnormal composition of the GBM leads to secondary events, resulting in renal fibrosis [13–15]. Important end points of the present study with AVE7688 included lifespan until death from progressive renal failure of treated mice versus untreated mice, systemic blood pressure, proteinuria, renal function, glomerular and tubulointerstitial fibrosis, inflammation, and profibrotic cytokines.

METHODS

Animals

Genotyping of COL4A3 −/− mice (Jackson Lab, Bar Harbor, ME, USA) was carried out by polymerase chain reaction (PCR) as described before [13, 14]. Treatment protocols for the mice were previously approved by local German authorities and supervised by veterinarians. Mice were bred on a 129/SvJ genetic background in a pathogen-free environment.

Experiments

Systolic arterial blood pressure was measured using a noninvasive pressure cuff system (LE5001, Panlab, Barcelona, Spain). Ten microliters of urine were used for microelectrophoresis on a gradient polyacrylamide gel, a semiquantitative technique used to qualify and quantify proteinuria [14, 15, 18]. Gels were stained by coomassie blue and analyzed by densitometry. Serum urea levels were analyzed on a Hitachi 917 Automatic Analyzer (Boehringer Mannheim, Mannheim, Germany).

AVE7688 was mixed into pallets of standard Western diet at a concentration of 125 parts per million according to recommendations by the manufacturer (Aventis GmbH, Bad Soden, Germany). Food intake was measured in metabolic cages to ensure constant drug intake. Wild-type mice were served as control animals. Eighty-four homozygous COL4A3 −/− mice were divided into 3 groups: (1) untreated animals (total $N = 40$; $N = 24$ survival; $N = 8$ sacrificed in week 7.5; $N = 8$ sacrificed in week 9.5); (2) early AVE7688 therapy, starting at week 4 before onset of proteinuria (total $N = 26$; $N = 10$ survival; $N = 8$ sacrificed in week 7.5; $N = 8$ sacrificed in week 9.5); (3) late AVE7688 therapy, starting at week 7 after onset of proteinuria (total $N = 18$; $N = 10$ survival; $N = 8$ sacrificed in week 9.5).

Light and electron microscopy

Eight animals per group were sacrificed at week 7.5, and 8 at week 9.5. Three mice per group were perfused transcardially, and kidneys were immersion-fixed for electron microscopy as described before [14]. Sections were taken on a Reichert Ultracut UCT ultramicrotome. A Zeiss Axiophot microscope (Zeiss, Göttingen, Germany) and a Zeiss EM 902 microscope were used for histologic documentation. For paraffin sections, kidneys were fixed as described before [14]. Sections were incubated overnight with primary antibodies rabbit anti-EHS-laminin (gift from M. Paulsson, Cologne, Germany), goat antifibronectin (St. Cruz, Heidelberg, Germany), and POX-conjugated mouse anti-α-smooth muscle actin (Dako, Hamburg, Germany). As negative control, slides were incubated with control immunoglobulin. Subsequently, cy3-labeled secondary antibodies (Jackson ImmunoReagents, West Grove, PA, USA) were added for 1 hour. Immunohistochemistry for CD3-positive T cells (rat anti-CD3; Serotec, Raleigh, NC, USA) and F4/80 positive monocytes/macrophages (rat anti-F4/80, Serotec, Raleigh, NC) was performed as described previously [19]. Endogenous peroxidase was blocked, and antigen retrieval was performed either in Antigen Retrieval Solution (Vector, Burlingame, CA, USA) by autoclaving (for CD3), or by proteinase K digestion (for F4/80). The Avidin/Biotin blocking Kit (Vector) was used to block endogeneous biotin before incubation with the primary antibodies. This was followed by incubation with a biotinylated antirat IgG antibody (Vector), and the ABC reagent (Vector). 3′,3′-Diaminobenzidine (DAB; Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black color product) served as detection system. Slides were counterstained with methyl green, dehydrated, and mounted.

Immunoblot

Thirty μg protein aliquots [as shown by BCA protein assay (Pierce, Rockford, IL, USA)] of tissue extracts from kidneys of untreated animals in week 7.5 and 9.5, and from kidneys of treated animals in week 7.5 and 9.5 were dissolved in sodium dodecyl sulfate (SDS) sample buffer, separated by electrophoresis in a 15% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and blocked. Mouse anti-TGFβ1 (R&D Systems, Minneapolis, MN, USA) and rabbit anti-CTGF
Each immunoblot was repeated 3 times. Protein-expression was analyzed densitometrically using a gel-pro analyzer developed using chemiluminescence. Protein-expression horseradish peroxidase (HRP; Dako), and the blot was incubated with secondary antibody conjugated with (Abcam Limited, Cambridge, United Kingdom) were incubated for 60 minutes. The membrane was then incubated with secondary antibody conjugated with horseradish peroxidase (HRP; Dako), and the blot was developed using chemiluminescence. Protein-expression was analyzed densitometrically using a gel-pro analyzer software (Media Cybernetics, San Diego, CA, USA). Each immunoblot was repeated 3 times.

**Statistics**

Data are presented as mean ± SEM. Data were analyzed by log-rank statistic (survival analysis) and two-way analysis of variance (ANOVA).

**RESULTS**

**AVE7688 extends lifespan of COL4A3 −/− mice**

Lifespan was continuously documented over a 12-month period (Fig. 1). No animals were lost due to infections or adverse effects of therapy. No signs of severe hypertension such as heart hypertrophy, increased septum size, or media sclerosis were found in any animals.

Lifespan of 24 untreated COL4A3 −/− mice was 70.9 ± 6.0 days. Life expectancy of mice in group II (early therapy; N = 10) increased by 143% to 172 ± 19 days (P < 0.001). Lifespan of mice in group III (late therapy; N = 10) increased by 53% to 109 ± 15 days (P < 0.01). The difference between early and late treatment was highly significant (P < 0.001).

**Both therapeutic regimens decrease systolic blood pressure and reduce proteinuria**

Mean systolic blood pressure was moderately increased in untreated COL4A3 −/− compared to wild-type control animals in week 9.5; however, differences were not significant in Student t test (Table 1). Both therapeutic regimens slightly reduced blood pressure in week 9.5 and 12 (data not significant). No differences in systemic blood pressure were found between the early and late treatment group.

Early therapy reduced proteinuria from 5 g/L in untreated animals to less than 1 g/L in week 7.5, and from 12 g/L to 2 g/L in week 9.5; late therapy reduced proteinuria to 4 g/L in week 9.5 (P < 0.05; Fig. 2). Reduction of proteinuria was more than 50% in both therapeutic regimens, with no significant differences between early and late treatment group after week 9.5.

Both therapies delay onset of uremia; however, deterioration of renal function is significantly slower in the early therapy group compared to late therapy after onset of proteinuria. In untreated mice (N = 6), serum urea started to rise above 50 mg/dL at week 7.5 and to 247 ± 27 mg/dL by week 9.5, followed by death soon after. In early treated mice (N = 6), deviation of urea was delayed by 4 weeks. By week 12, urea of early treated mice rose to 67 ± 11 mg/dL; in contrast, urea of late treated mice (N = 6) was significantly higher (153 ± 21; P < 0.05).

Early and late AVE7688 therapy reduces abnormal deposition of the renal extracellular matrix, as well as numbers of activated fibroblasts, T lymphocytes, and macrophages. Electron microscopy of COL4A3 −/− mice showed characteristic thickening and splitting of the GBM in untreated animals (Fig. 4E). Abnormal intracellular amounts of fibrillar collagens, and a complete loss of the podocyte foot processes in untreated animals, could also be observed (Fig. 4E). These changes appeared to be improved by early AVE7688 therapy (Fig. 4I) because intact foot processes and slit membranes could still be found.

Light microscopy demonstrated early periglomerular fibrosis in untreated mice by week 7.5 (Fig. 4F), leading to complete loss of glomerular function and nephrons.

### Table 1. Antihypertensive effect of AVE7688

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Wild-type placebo</th>
<th>Homozygous late therapy</th>
<th>Homozygous early therapy</th>
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<tr>
<td>9.5</td>
<td>111 ± 9</td>
<td>116 ± 14</td>
<td>105 ± 13</td>
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<tr>
<td>12</td>
<td>113 ± 11</td>
<td>animals dead</td>
<td>111 ± 16</td>
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<td>N = 5</td>
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Systolic mean arterial blood pressure (mm Hg) of COL4A3 −/− mice with and without medical treatment.
paralleled by severe glomerular and tubulointerstitial fibrosis in week 9.5 (Figs. 4G and H). Fibrotic changes of the glomerulum, as well as of the tubulointerstitium, were less severe in the early therapy group in week 7.5 (Fig. 4J). In these animals, a preserved glomerular architecture without apparent tubulointerstitial fibrosis could be noted in week 9.5 (Figs. 4K and L), with only mild mesangial expansion (Fig. 4K).

Immunohistochemistry confirmed these changes (Fig. 5): wild-type mice showed scant staining for fibronectin (Fig. 5A). Fibronectin staining was increased in the periglomerular matrix of untreated COL4A3 −/−, a typical finding of severe glomerulosclerosis (Fig. 5B). Compared to untreated COL4A3 −/− mice, staining was reduced in early treated animals (Fig. 5D), as well as in animals treated after onset of proteinuria (late therapy) (Fig. 5C), both treatment groups showed only a mild staining of the periglomerular matrix.

Healthy control animals showed a thin tubular and glomerular basement membrane (EHS-laminin; Fig. 5E and I). In contrast, increased matrix deposition was seen in untreated animals (Fig. 5F and J), laminin staining being present in the intra- and periglomerular, as well as in intertubular regions. Localized shrinkage of tubular lumen was also noted, indicating loss of function of different nephrons. Periglomerular and intertubular signal was decreased in the early therapy group, with almost normal staining in the intertubular space and a preserved tubular lumen, suggesting preserved function of nephrons (Fig. 5G and K). Signalling was more prominent and, again, no loss of nephrons was found in the late therapy group (Fig. 5H and L), indicating that late therapy after onset of proteinuria prevented periglomerular and tubulointerstitial fibrosis, as well.

The anti-inflammatory potential of AVE7688 therapy was investigated by immunostaining for T lymphocytes (Fig. 6A to D), macrophages (Fig. 6E to H), and activated fibroblasts (Fig. 6I to L): compared to wild-type control animals (Fig. 6A), untreated COL4A3 −/− showed a glomerular, periglomerular, and tubulointerstitial signal for CD3-positive cells (T-lymphocytes, Fig. 6B). This signalling was reduced by late (Fig. 6C) and early therapy (Fig. 6D). Untreated COL4A3 −/− mice also showed a periglomerular and tubulointerstitial signal for F4/80-positive cells (macrophages, Fig. 6F, wild-type
Fig. 4. Nephroprotective effect of AVE7688 therapy on renal architecture. Representative figures from wild-type control animals (upper row, A to D), untreated (middle row, E to H), and AVE7688 early treated COL4A3 −/− mice (lower row, I to L). A, B, E, F, I, and J: all mice 7.5 weeks of age; C, D, G, H, K, and L: all mice 9.5 weeks of age. Original magnification, left row: 20,000- to 30,000-fold; middle row: 1000-fold; right row: 400- to 600-fold. C, capillary lumen; P, podocytes and podocytes’ foot processes.

Fig. 5. Antifibrotic effect of AVE7688 therapy. Immunostaining for fibronectin and EHS-laminin. Representative figures from wild-type control animals (left, A, E, and I), untreated (B, F, and J), early treated (C, G, and K), and late treated COL4A3 −/− mice (D, H, and L). All mice 9.5 weeks of age. Original magnification, upper row: 400-fold; middle row: 100-fold; lower row: 800-fold.
Fig. 6. Anti-inflammatory effect of AVE7688 therapy. Immunostaining for T lymphocytes (upper row), activated macrophages (middle row), and fibroblasts (lower row). Representative figures from wild-type control (A, E, and J), untreated (B, F, and J), late treated (C, G, and K), and early treated COL4A3 −/− mice (D, H, and L). All mice 9.5 weeks of age. Original magnification, 100- and 400-fold.

Fig. 7. Antifibrotic effect of AVE7688 therapy. Immunoblot for TGFβ1 and CTGF. IOD, integrated optical density; N, number of animals pooled.

control, Fig. 6E). Again, this signalling was reduced by late (Fig. 6G) and early therapy (Fig. 6H). Similar results were found by staining for α-smooth muscle actin (activated fibroblasts, wild-type control, Fig. 6I). Untreated COL4A3 −/− mice showed a periglomerular and tubulointerstitial signal (Fig. 6J) that was reduced by late and early AVE7688 therapy (Fig. 6K and L).

For further analysis, glomeruli of 3 different animals of each group were evaluated for glomerulosclerosis in week 9.5 by a blinded observer. Glomerulosclerosis was defined as loss of more than 50% of glomerular lumen due to extracellular matrix accumulation. Two out of 94 (2.1%) glomeruli of healthy control animals showed sclerosis, whereas 78 out of 87 (89.7%) did in untreated COL4A3 −/−. In contrast, only 12 out of 92 (13.0%) of glomeruli of early AVE7688-treated COL4A3 −/−, and 28 out of 88 (31.8%) of glomeruli of late-treated animals showed sclerosis. Tubulointerstitial fibrosis was evaluated in a similar manner by grading 12 different kidney sections of 3 animals per experimental group (total of 36 sections) into zero, 1+, and 2+ accumulation of extracellular matrix by a blinded observer. Healthy control animals showed no accumulation of extracellular matrix in any section (matrix score 0.0); untreated COL4A3 −/− showed an average matrix score of 1.86. Early treated mice showed a matrix score of 0.47, late treated mice of 0.81.

AVE7688 therapy strongly reduces TGFβ1 and CTGF protein expression in this model of progressive renal fibrosis. We speculated that the nephroprotective effect of AVE7688 in the COL4A3 −/− mice may be due to down-regulation of the profibrotic cytokines TGFβ and CTGF (Fig. 7). Compared to untreated mice in week 7.5 and 9.5 (lanes 5 and 2), early AVE7688 therapy (lanes 6 and 3) resulted in down-regulation of TGFβ (by nearly 300% and of CTGF by more than 300% (This effect was found to be weaker in the late therapy group (lane 1) (down-regulation of TGFβ and CTGF by nearly 50%).

DISCUSSION

COL4A3 −/− mice with Alport syndrome serve as a model of progressive renal disease, leading to renal fibrosis and end-stage renal failure [16]. All untreated
animals showed a similar onset of uremia and proteinuria, resulting in death by 10 weeks of age. These changes allowed observation of the antifibrotic, anti-inflammatory nephroprotective potential of the novel vasopeptidase inhibitor AVE7688. The drug was given before onset of proteinuria (early therapy, mimicking the clinical setting of a prophylactic nephroprotective therapy in patients with microalbuminuria), or after onset of proteinuria above 3 g/L (late therapy, mimicking the clinical setting of a nephroprotective therapy in patients with nephrotic syndrome and ongoing renal insufficiency). A nephroprotective effect of both therapeutic regimens was clearly demonstrated: lifespan—the most evident end point—was prolonged by 143% in the early therapy group and by 53% in the late therapy group. A significantly greater effect on lifespan was demonstrated when therapy started early as compared to the late therapy.

The antihypertensive effect of AVE7688 (Table 1) in our non- or only mild-hypertensive animal model with Alport disease [20] was mild, and not significantly different to treatment with an AT1 antagonist that showed similar antihypertensive, but a weaker nephroprotective, antifibrotic potential than AVE7688 in the same mouse model in a previous study [15]. Therefore, the strong nephroprotective effect of AVE7688 cannot be explained by its antihypertensive effects alone.

Urinary proteins have been shown to induce progressive interstitial fibrosis, and the known antiproteinuric effects of ACE inhibitors and AT1 antagonists have been suggested to be nephroprotective [21–23]. Our study demonstrated a strong antiproteinuric effect of early and late AVE7688 therapy that may well have contributed to renoprotection in this model. Both therapeutic regimens showed a comparable antiproteinuric potential. However, early therapy was significantly more effective in prolonging lifespan; therefore, the better nephroprotective effect of early AVE7688 therapy might be explained by additional nephroprotective mechanisms beyond its antiproteinuric and antihypertensive action.

ACE inhibitors have been shown to be effective in preserving renal function in several human renal diseases [2–4, 21–23]. ACE inhibitors block the conversion of angiotensin I to angiotensin II, a growth factor which activates fibroblasts, leading to increased synthesis of matrix proteins [24]. Angiotensin II is also a profibrotic cytokine, activating mononuclear cells and increasing proinflammatory mediators [24], as well as regulating matrix degradation. Some of the downstream effects of angiotensin II are mediated directly via the TGFβ pathway, which has been shown to be important in the pathogenesis of renal fibrosis and inflammatory cell infiltration such as monocytes in AS [13–15, 25, 32]. Our data show that levels of TGFβ and CTGF are increased in untreated COL4A3 −/− mice. Early therapy with AVE7688 reduced TGFβ and CTGF expression more efficiently than late therapy did. This was paralleled by the preservation of renal function. These results emphasize the role of TGFβ and CTGF in progression of renal fibrosis in the COL4A3 −/− mice.

Angiotensin II plays an important role in chemotaxis of inflammatory cells such as macrophages and monocytes and additional proinflammatory effects [26]. Our model of chronic renal fibrosis is not a primary inflammatory animal model. However, kidneys of untreated mice showed a profound infiltration of inflammatory cells, such as T lymphocytes, macrophages, as well as an increased number of activated fibroblasts. COL4A3 −/− mice treated with AVE7688 showed an almost complete loss of these inflammatory infiltrates (Fig. 6), which was superior to the anti-inflammatory effect of ramipril (data not shown). Inflammatory cells play a major role in developing tubulointerstitial fibrosis and loss of nephron function, and have as well been shown to be key participants in the pathogenesis of renal fibrosis in AS [15, 27] and in most other chronic renal diseases leading to fibrosis and end-stage renal failure.

In previous studies with COL4A3 −/− mice, we found an antifibrotic effect of early therapy with the ACE inhibitor ramipril, which prolonged lifespan by 111% [14]. Late therapy with ramipril, however, did not prolong lifespan in COL4A3 −/− mice. In contrast, late therapy with AVE7688 prolonged lifespan by 53% (P < 0.01 compared to placebo). The superior nephroprotective potential of the vasopeptidase inhibitor might be explained by its anti-inflammatory effects (Fig. 6).

CONCLUSION

Lifespan of Alport mice strongly depended on the time point when AVE7688 treatment was initiated. AVE7688 might, as well, be able to delay onset of renal failure and fibrosis in humans with Alport syndrome. We conclude that therapy with AVE7688 in patients with chronic renal disease might have a profound nephroprotective effect in regards to reduction of both proinflammatory and profibrotic factors, being the major players in progression of chronic renal diseases. The nephroprotective potential of AVE7688 might be most effective in patients with yet mild chronic renal disease (such as microalbuminuria), but might still be profound in patients with progressed chronic renal disease (such as patients with nephrotic syndrome or beginning renal failure). Previous studies showed that vasopeptidase inhibition could prevent diabetic nephropathy [12, 28, 29] and endothelial dysfunction [11, 30, 31]. According to our data, the novel vasopeptidase inhibitor AVE7688 might indeed have superior effects over pure ACE inhibitors in regards to prevention and delay of renal end-organ damage due to its additive antifibrotic and anti-inflammatory potential.
REFERENCES


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