Original Article

Pirfenidone and candesartan ameliorate morphological damage in mild chronic anti-GBM nephritis in rats

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Abstract

Background. The antifibrotic substance pirfenidone and the angiotensin II type I receptor antagonist candesartan cilexetil, given alone and in combination, were tested in rats with chronic anti-glomerular basement membrane glomerulonephritis (anti-GBM GN).

Methods. Male Wistar rats with anti-GBM GN were treated for 8 weeks with candesartan (4 mg/kg body weight/day), pirfenidone (500 mg/kg body weight/day) or a combination of both drugs. One GN group received no treatment and untreated non-GN-rats were used as controls. Blood pressure and urinary protein excretion were measured after 3 and 7 weeks. Kidney histology was complemented by ultrastructural investigation and by quantification of collagen Iα mRNA.

Results. The percentage of glomeruli with adsorption droplets in podocytes correlated well with the amount of proteinuria (r = 0.873, P < 0.01) and was significantly lowered in rats treated with candesartan (8.3 vs GN 24.6%), pirfenidone (9.8%) and combined treatment (2.6%, P < 0.05 vs candesartan alone). A comparable lowering was seen for segmental sclerosis (GN 11%, candesartan 0.7%, P < 0.05 vs GN, pirfenidone 1.8%, P = 0.09 vs GN, candesartan/pirfenidone 0.1%, P > 0.5 vs candesartan alone). Cortical collagen Iα mRNA expression was significantly decreased in all treatment groups. Ultrastructural investigation showed an amelioration of basement membrane alterations and podocyte damage in the treatment groups. Candesartan caused significant blood pressure reduction and the effect was significantly enhanced by combination therapy after 3 weeks. Rats treated with pirfenidone showed blood pressure values similar to control rats.

Conclusion. Pirfenidone has a beneficial effect on morphological changes in anti-GBM GN comparable with candesartan although with a trend to slightly better results with candesartan treatment. Moreover, our results suggest an additive effect of combination treatment.

Keywords: candesartan; glomerulonephritis; pirfenidone; podocytes; segmental sclerosis

Introduction

Inhibitors of the renin angiotensin system are known to be renoprotective. This has been proven in several animal models of progressive renal damage with the use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type I (AT1) receptor antagonists (reviewed in [1]). Kidney functionality, as shown by the degree of proteinuria, is preserved in a better way, and morphological signs of irreversible damage such as glomerulosclerosis, tubular atrophy and interstitial fibrosis are reduced by both ACE inhibitors and AT1 receptor antagonists. Although inhibitors of the renin–angiotensin system are capable of slowing down development of renal failure, they frequently do not arrest or reverse decline of kidney functional parameters such as proteinuria and glomerular filtration rate in the long run. Combining inhibitors of the renin–angiotensin system with renoprotective drugs with other mechanisms of action might preserve kidney function in a superior way.

Pirfenidone is a new antifibrotic drug. Its antifibrotic effect was first described in 1995 in a hamster model of bleomycin-induced lung fibrosis [2] and was later confirmed in several animal models with fibrosing diseases in different organs. Pirfenidone has not only antifibrotic but also anti-inflammatory properties. It reduced lethality in a murine model of endotoxin shock [3] and attenuated ischaemia–reperfusion injury in the rat small intestine [4]. Its mechanism of action...
is poorly understood. Interference with reactive oxygen species [2], suppression of pro-inflammatory factors such as tumour necrosis factor-α [3] and reduction of transforming growth factor-β (TGF-β) production are reported as possible working mechanisms [5].

Pirfenidone has been tested in several rat models of kidney disease focusing on development of tubular damage and interstitial fibrosis.

Studies of pirfenidone in glomerulonephritis (GN) are scarce. Pirfenidone reduced proteinuria and glomerular damage in anti-Thy-1 nephritis with acceleration of kidney damage by uninephrectomy [6]. There is no study comparing pirfenidone with other renoprotective drugs in GN.

Anti-glomerular basement membrane (anti-GBM) GN is a rat GN model with marked GBM damage comparable with crescentic GN in man. In this model, focal capillary wall destruction, podocyte damage and crescent formation arise in the early phase, and later focal and segmental sclerosis associated with tubular damage and interstitial fibrosis develops.

The purpose of the present study was to prove the long-term renoprotective effects of pirfenidone in anti-GBM GN, to compare possible beneficial effects of pirfenidone in anti-GBM GN with the effect of the AT1 receptor antagonist candesartan and to investigate a potentially additive effect of combination therapy.

Our working hypothesis was that pirfenidone may have a beneficial effect on functional and morphological parameters comparable with candesartan, and that an additive effect of combined treatment due to different mechanism might be present.

Materials and methods

All experiments were performed in accordance with and under approval of the Norwegian State Board for Biological Experiments with Living Animals at the University of Bergen, Bergen, Norway.

Animals

Male Wistar rats \( (n=48) \) with a body weight range of 224–276 g were purchased from Møllergaard, Denmark. The rats were kept three in each cage at stable room temperature, and at a regular 12 h dark and light rhythm. They were fed standard rat chow, containing 0.30% sodium, 0.70% potassium, 0.88% calcium and 18% crude protein, and had free access to water. The rats had 2 weeks to adapt to the new environment before the start of the experiments. For urine collection, each rat was placed in a metabolic cage for 24 h.

Design of experiment

The rats were divided into five groups: (i) normal controls \( (n=8) \) without GN and without treatment; (ii) anti-GBM GN \( (n=10) \) without treatment; (iii) anti-GBM GN treated with candesartan \( (n=10) \); (iv) anti-GBM GN treated with pirfenidone \( (n=10) \); and (v) anti-GBM GN treated with both candesartan and pirfenidone \( (n=10) \).

Induction of anti-GBM GN

Antibodies against rat GBM were produced by repeated intraperitoneal injections of purified rat GBM in rabbits as previously described [7]. Samples of venous blood (10 ml) were taken from the rabbits every 3–4 weeks. Serum was separated by centrifugation and stored in a pool at \(-20^\circ C\) until later use. To induce GN, a single dose of 1.5 ml of pooled serum was injected intravenously into each rat.

Measurements

The systolic blood pressure was measured with the tail cuff method, using an automatic recorder (Ugo Basile, Comerio, Italy), in awake animals prior to antiserum injection, and 3 and 7 weeks after antiserum injection. The animals were customized to blood pressure measurements before the start of the experiment, and the blood pressure value was calculated as the average value of three different measurements.

Body weights were recorded at the same time as blood pressure measurements.

Urine protein excretion was measured in 24 h urine samples at the start and at week 3 and 7 by using a U/CSF protein assay kit (Roche/Hitachi 704 end-point assay; Roche Diagnostics GmbH, Mannheim, Germany).

Treatments

Candesartan cilexetil (Astra-Zeneca, Sweden) was given at a daily dose of 4.0 mg/kg body weight and pirfenidone (Marnac Inc., Dallas, TX) at a daily dose of 500 mg/kg body weight in a watery solution (1 ml) by gastric gavages. In the group with combined treatment, candesartan and pirfenidone were given in a single gavage (1 ml) in the same concentrations as mentioned above. Treatments started on the second day after antiserum injection and continued for 8 weeks.

End of the experiment

The rats were anaesthetized with pentobarbital (50 mg/ml, 0.15 ml/100 mg body weight). Blood was collected from the aorta.

The first 3 ml of blood were taken into pre-chilled K-EDTA tubes (4°C). Plasma was separated by centrifugation for 5 min at 3000 r.p.m. at 4°C and stored at \(-20^\circ C\) until later analysis for plasma renin activity by radioimmunoassay [8]. Thereafter, a sample of 4–5 ml of blood was taken into tubes and centrifuged for 5 min at 3000 r.p.m. The serum was stored at \(-20^\circ C\) for later measurements of serum electrolytes, creatinine and albumin concentrations.

The right kidney was perfused \textit{in vivo} via the abdominal aorta with 4% buffered formaldehyde and embedded in paraffin by standard procedures. Frontal sections were stained with haematoxylin and eosin, periodic acid–Schiff (PAS) and Lendrum’s MSB.

Small pieces from the cortex of the left kidney were fixed in 2% buffered glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in EPON. Semi-thin sections were stained with toluidine blue. Blocks with representative changes were chosen for ultrastructural investigation. Ultrathin sections were stained with uranyl acetate and lead citrate, and studied in a Jeol 100 CX electron microscope.
A thin slice of renal cortex from the left kidney was fixed in RNA-later solution (Ambion Inc., Austin, TX), as recommended by the manufacturer, for later quantification of mRNA for collagen Iα.

**Light microscopy**

PAS-stained sections underwent microscopic examination regarding glomerular, tubulointerstitial and vascular changes. All microscopic and morphometric investigations were performed in a blinded manner.

A central cortical area of the frontal plane, located symmetrically on both sides of an imaginary transversal axis through the papillary tip, was marked. This region extended from the capsule to the corticomedullar border and contained 100 glomeruli. The glomeruli were investigated with regard to podocyte changes (adsorption droplets) and segmental sclerosis. The same area assumed to contain 100 nephrons was examined with regard to tubular changes and interstitial inflammation. The number of tubular cross-sections with signs of degeneration was counted and graded according to Table 1. Degenerated tubules were defined as tubules with thickened basement membrane. Interstitial inflammation was assessed semi-quantitatively as described in Table 1.

**Morphometry**

Morphometry was performed with the point counting method. Digital images from cortical tissue were assessed by a systematic uniform random procedure. Images were taken with a 20× objective. The resolution of the images was 1500 x 1500 pixels. Using a point grid with 100 points, a total of 1000 points per kidney was analysed. Volume fractions were estimated counting the points hitting the phases of interest divided by the points hitting the reference space. The volume of the kidney cortex was defined as the reference space. Assuming the specific weight of the kidney tissue equals 1 and assuming the volume fraction of the reference space. Fractions were estimated counting the points hitting the phases of interest divided by the points hitting the reference space. The volume of the kidney cortex was defined as the reference space. Assuming the specific weight of the kidney tissue equals 1 and assuming the volume fraction of the medulla comprises 30% of the total kidney volume [9], the tissue equals 1 and assuming the volume fraction of the reference space. Assuming the specific weight of the kidney phases of interest divided by the points hitting the reference space. Fractions were estimated counting the points hitting the phases of interest divided by the points hitting the reference space.

\[
V(\text{cortex/ml}) = \frac{W_{\text{kidney(g)}} \cdot 0.7}{1 \cdot (\text{g/ml})}
\]

Phases of interest were tubules, interstitium and capillaries. Basement membranes of tubules were counted as interstitium. The absolute volumes of the phases of interest \(V(Y)\) were calculated after equation 2:

\[
V(Y) = \frac{\sum P(Y)}{\sum P(\text{ref})}
\]

\(P(\text{ref})\) are the points hitting the reference space and \(P(Y)\) are the points hitting the phase of interest.

To determine how many fields have to be analysed, a preliminary study was done: cumulative means were plotted after each field until the means remained steady [10].

The coefficient of error for volume fractions in each animal was calculated after equation 3, where there are \(k\) images and each summation is over 1 to \(k\) [11]:

\[
c_{\text{est}}CE(V_\text{Y}) = \left[ \frac{k}{k-1} \left( \frac{\sum P(\text{ref})^2}{\sum P(\text{ref})} + \frac{\sum P(Y)^2}{\sum P(Y)} - 2 \frac{\sum P(\text{ref}) P(Y)}{\sum P(\text{ref}) \sum P(Y)} \right) \right]^{1/2}
\]

The mean coefficient of error for volume fractions was 3.9 for tubules, 9.8 for interstitium and 21.5 for capillaries.

**Collagen Iα mRNA quantification**

Total RNA was purified from renal cortical pieces using TRIzol following the manual from Gibco (Invitrogen, Carlsbad, CA).

Quantification of collagen Iα mRNA was done by real-time reverse transcription–polymerase chain reaction (RT–PCR). First strand cDNA was synthesized from isolated total RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) and primed by pd(N)6 primers. Primers for RT–PCR amplification of collagen Iα were selected for a 76 bp fragment containing the splicing site of two exons. The forward primer was 5′-ACC TACAGCACCGCTTGTGGAT-3′, and the reverse primer was 5′-GTTGGTTTGTATTGGATGACTGTTT-3′. The taqman probe was 5′-CTGCACCGGATCACCACGGGACTTGG-3′, marked with FAM and 3′-TAMRA. The amplified collagen Iα cDNA was normalized against amplified 18S rRNA to compensate for any changes due to RNA degradation, reverse transcriptase efficiency or amplification success. The primers were made for a 68 bp fragment. The forward primer was 5′-AGTCCCTGCCCCTTTGTACA-3′, and the reverse primer was 5′-GATCCGGGCGCTCACATA AAC-3′. The taqman probe was 5′-CGCCCGCTGCGT ACTACCGATTGG-3′, marked with 5′-Yakima Yellow and 3′-TAMRA. Primers and probes were constructed using Primer Express (Applied Biosystems).

Renal tissue from one normal rat was used as standard in the analyses of the amplification products. The collagen Iα expression of this animal was set to 100% and the collagen Iα expression of all other animals was then compared with this. RT–PCR was performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using a qPCR™ Core Kit (Eurogentec, Seraing, Belgium). Primer concentrations were optimized before use in quantification. Forward primers for both collagen Iα and 18S rRNA were used in a final concentration of 0.3 μM. Reverse primers for collagen Iα and 18S rRNA were used in a final concentration of 0.9 μM.

For each sample, 1 μg of total RNA in 15 μl was used for the cDNA synthesis. In each amplification reaction, 1 μl

<table>
<thead>
<tr>
<th>Table 1. Grading of tubular degeneration and interstitial inflammation</th>
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<tbody>
<tr>
<td>No. of degenerated tubules/predefined area</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>1–10</td>
</tr>
<tr>
<td>11–50</td>
</tr>
<tr>
<td>51–100</td>
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<td>&gt;100</td>
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of cDNA solution was used as template. All amplifications of both collagen I and 18S RNA were done using three parallel amplification reactions. The amplifications were performed under standard ABI conditions using 19.0 µl reaction volumes.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections. The following antibodies were used: ED1 (CD68-like, Serotec UK, 1:1000), W3/13 (CD43, Serotec, 1:1000) and anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY; 1:3200). ED1 stains monocytes and macrophages, W3/13 stains T lymphocytes, plasma cells and granulocytes, and nitrotyrosine provides evidence for the formation of peroxynitrite. The staining protocol was optimized for the different antibodies. Sections for staining with ED1 and W3/13 were pre-incubated in blocking serum (2.5% goat serum and 2.5% rat serum) for 1 h. Microwave heat-induced antigen retrieval was done for 15 min in citrate buffer pH 6 (ED1 and W3/13) or Tris EDTA buffer pH 9 (nitrotyrosine). Endogenous peroxidase was blocked with peroxidase blocking reagent containing hydrogen peroxide and 15 mM sodium azide (DAKO Corporation, Carpinteria, CA). Sections were incubated with primary antibodies overnight at 4°C, followed by incubation with enzyme- and antibody-marked polymer (DAKO EnVision, goat anti-rabbit for nitrotyrosine and goat anti-mouse for ED1 and W3/13). Diaminobenzidine was used for visualization of immunoreactivity, followed by haematoxylin for nuclear counterstaining.

Negative controls were performed by omitting the primary antibody. In the case of nitrotyrosine stain, an additional negative control was carried out by pre-incubating the antibody with a 10 mM nitrotyrosine solution pH 7.4 for 1 h at room temperature. The amount of nitrotyrosine positivity was assessed by semi-quantitative grading of the number of positive cells and the degree of staining intensity (grade 1–5, grade 5 maximal expression). For quantification of ED1 and W3/13, positive cells were counted in 30 consecutive glomeruli and 20 consecutive high power fields (HPFs; magnification 400×) of kidney cortex.

**Statistical analysis**

Data are expressed as mean ± SE. Group differences were assessed by the χ² (if control subjects had the value 0), the Wilcoxon–Mann–Whitney test (exact tests using Monte Carlo), two-sample t-tests and one-way analysis of variance (ANOVA) with Scheffe post hoc tests. In cases of unequal variances, Tamhane post hoc tests were used. Correlation analysis between podocyte changes (adsorption droplets) and proteinuria was done by Spearman’s rank correlation. Statistical calculations were performed with SPSS 11.0. P < 0.05 was considered significant.

**Results**

**Proteinuria**

Rats with untreated GN had a protein excretion of 191 ± 45 mg/24 h after 3 weeks, P < 0.01 vs control, and 157 ± 41 mg/24 h after 7 weeks, P < 0.001 vs control (Figure 1). Candesartan reduced proteinuria to 90 ± 29 mg/24 h at week 3 and to 57 ± 23 mg/24 h at week 7 (P < 0.05 vs GN). Pirfenidone treatment also caused a decline in urinary protein excretion to 118 ± 46 mg/24 h at week 3 (P < 0.08 vs GN), and 88 ± 40 mg/24 h at week 7 (P < 0.05 vs GN). Proteinuria was reduced further by combination treatment to 67 ± 24 mg/24 h at week 3 and to 38 ± 18 mg/24 h at week 7, though not significantly different from monotherapy with candesartan (P > 0.5 week 3 and P > 0.7 week 7).

**Blood pressure**

The mean systolic blood pressure of all animals was 150 ± 2 mmHg at the start of the experiment (Figure 2). While blood pressure in control rats was 144 ± 5 mmHg at week 3 and 145 ± 4 mmHg at week 7, there was a slight numerical increase in rats with GN, being 154 ± 19 mmHg at week 3 (P > 0.9 vs control) and 152 ± 15 mmHg at week 7 (P > 0.9 vs control). Blood pressure in rats treated with pirfenidone remained at values similar to those of control rats. Blood pressure in candesartan-treated rats decreased compared with the start and with the untreated GN rats at both weeks 3 and 7, being 127 ± 2 mmHg at week 3 (P < 0.05 vs GN rats) and 122 ± 5 mmHg at week 7 (P < 0.01 vs GN rats). An additional decrease in blood pressure was observed in rats treated with the combination of candesartan and pirfenidone to 113 ± 3 mmHg at week 3 (P < 0.05 vs candesartan group) and 115 ± 3 mmHg at week 7 (P > 0.9 vs candesartan group).

**Body weight**

Control rats had the highest body weight during the study, and weighed 404 ± 12 g at the end of the study. Generally body weight in untreated and treated GN rats was numerically reduced, being 387 ± 13 g...
Pirfenidone and candesartan in glomerulonephritis

Values are given as mean±SE. *P<0.05 vs GN, **P<0.01 vs Cd (ANOVA).

Table 2. Effect of treatment on serum parameters: urea nitrogen (urea-N), serum creatinine, serum albumin concentrations and plasma renin activity (PRA)

<table>
<thead>
<tr>
<th></th>
<th>Serum urea-N (mmol/l)</th>
<th>Serum creatinine (μmol/l)</th>
<th>Serum albumin (mg/l)</th>
<th>Plasma renin activity (μg/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.95±0.29</td>
<td>55.1±2.77</td>
<td>36.3±0.31</td>
<td>13.8±2.81</td>
</tr>
<tr>
<td>GN</td>
<td>7.61±0.73</td>
<td>56.8±1.73</td>
<td>31.4±1.65*</td>
<td>6.6±1.38*</td>
</tr>
<tr>
<td>Cd</td>
<td>6.56±0.17</td>
<td>53.5±1.29</td>
<td>34.2±0.84</td>
<td>50.6±5.84**</td>
</tr>
<tr>
<td>Pfd</td>
<td>6.43±0.25</td>
<td>52.2±1.29</td>
<td>34.2±1.10</td>
<td>6.8±1.86*</td>
</tr>
<tr>
<td>Cd/Pfd</td>
<td>6.98±0.29</td>
<td>53.9±2.04</td>
<td>35.6±0.88</td>
<td>39.1±8.40**</td>
</tr>
</tbody>
</table>

GN = glomerulonephritis group; Cd = candesartan group; Pfd = pirfenidone group; Cd/Pfd = group with combination treatment. Values are given as mean±SE. *P<0.05 vs control; **P<0.001 vs control, GN and Pfd (Student t-test).

in untreated GN rats, 384±11 g in candesartan-treated rats and 389±11 g in pirfenidone-treated rats at the end of the study. The body weight of the rats getting combination treatment of pirfenidone and candesartan was the lowest at any time point of the study. The rats weighed 344±8 g at the end of the study (P<0.05 vs control rats).

Blood chemistry at the end of the study

Serum creatinine and urea nitrogen concentration did not change and did not differ among the groups at the end of the study (Table 2). Serum albumin concentration decreased in untreated GN rats, while it remained at control levels in treated rats. Plasma renin activity was significantly reduced in GN and pirfenidone-treated rats. Candesartan treatment—alone or in combination with pirfenidone—increased plasma renin activity significantly compared with controls (P<0.001).

Histological findings

Figure 3 shows representative changes in glomeruli, tubules and interstitium. The most prominent glomerular alteration in GN rats was segmental sclerosis (Figure 3e and f) accompanied by acute and chronic tubular damage, shown by tubular dilation, hyaline casts, detached epithelial cells in tubular lumina and tubular degeneration with thickened basement membranes and dedifferentiated epithelium. The interstitium was widened by both oedema and fibrosis, and there were spots of mononuclear infiltration (Figure 3d).

Glomerular and tubulointerstitial changes were clearly reduced in all treatment groups (Figure 3g–o). There were only few glomeruli with segmental sclerosis and the lesions were smaller compared with untreated GN rats. Glomeruli also showed focal mesangial and endocapillary hypercellularity, small segments with capillary wall thickening and hypertrophic podocytes with adsorption droplets and pseudocysts. Only few tubules were dilated or showed detached necrotic epithelial cells; likewise the number of hyaline cylinders had decreased considerably. There was little interstitial oedema, fibrosis or inflammation.

Figure 4 shows quantitative evaluation of these histological findings. Segmental sclerosis was seen in 11.0±4.8% of glomeruli in GN rats and was reduced to 0.7±0.5% by candesartan treatment (P<0.05 vs GN), 1.8±0.9% by pirfenidone treatment (P=0.09 vs GN) and 0.1±0.1% by combination treatment (P=0.51 vs candesartan alone). Whereas 24.6±3.6% of glomeruli showed podocytes with adsorption droplets in GN rats, only 8.3% of glomeruli in the candesartan-treated group (P<0.01 vs GN) and 9.8% of glomeruli in the pirfenidone-treated group (P<0.01 vs GN) revealed these altered podocytes. Combined treatment further reduced the percentage of glomeruli with adsorption droplets in podocytes to 2.6±2.6% (P<0.05 vs candesartan), thus indicating an additive effect of combination therapy. The percentage of glomeruli with adsorption droplets in podocytes correlated well with the amount of proteinuria (r=0.873, P<0.01).

The tubular degeneration score was 2.5±0.4 in the GN group. Tubular degeneration was significantly lower in candesartan-treated rats (score 0.7±0.3, P<0.01 vs GN), in pirfenidone-treated rats (score 1.0±0.5, P<0.05 vs GN) and in rats with combination treatment (0.4±0.2, P<0.05 vs candesartan treatment alone). Candesartan and combined treatment of candesartan/pirfenidone significantly ameliorated interstitial inflammation (score GN 1.8±0.3; candesartan treatment 0.8±0.2, P<0.05 vs GN; combination treatment 0.6±0.4, P<0.7 vs candesartan alone), whereas pirfenidone did not show significant preventive effects (0.9±0.3, P=0.07 vs GN).

Morphometric measurements of tubular, interstitial and post-glomerular capillary volumes are shown in Table 3. There was a significant increase of tubular volumes in GN rats compared with control rats. Treatment with candesartan and candesartan/pirfenidone reduced tubular enlargement significantly. Combination treatment could not significantly improve the effect of candesartan treatment alone. The decrease...

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**Figure 2.** Effect of treatment on systolic blood pressure after 3 and 7 weeks. GN = glomerulonephritis group; Cd = candesartan group; Pfd = pirfenidone group; Cd/Pfd = group with combination treatment. Values are shown as mean±SE; *P<0.05 vs GN, **P<0.01 vs Cd (ANOVA).

**Figure 3.** Representative changes in glomeruli, tubules and interstitium. The most prominent glomerular alteration in GN rats was segmental sclerosis (Figure 3e and f) accompanied by acute and chronic tubular damage, shown by tubular dilation, hyaline casts, detached epithelial cells in tubular lumina and tubular degeneration with thickened basement membranes and dedifferentiated epithelium. The interstitium was widened by both oedema and fibrosis, and there were spots of mononuclear infiltration (Figure 3d).

**Figure 4.** shows quantitative evaluation of these histological findings. Segmental sclerosis was seen in 11.0±4.8% of glomeruli in GN rats and was reduced to 0.7±0.5% by candesartan treatment (P<0.05 vs GN), 1.8±0.9% by pirfenidone treatment (P=0.09 vs GN) and 0.1±0.1% by combination treatment (P=0.51 vs candesartan alone). Whereas 24.6±3.6% of glomeruli showed podocytes with adsorption droplets in GN rats, only 8.3% of glomeruli in the candesartan-treated group (P<0.01 vs GN) and 9.8% of glomeruli in the pirfenidone-treated group (P<0.01 vs GN) revealed these altered podocytes. Combined treatment further reduced the percentage of glomeruli with adsorption droplets in podocytes to 2.6±2.6% (P<0.05 vs candesartan), thus indicating an additive effect of combination therapy. The percentage of glomeruli with adsorption droplets in podocytes correlated well with the amount of proteinuria (r=0.873, P<0.01).

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Morphometric measurements of tubular, interstitial and post-glomerular capillary volumes are shown in Table 3. There was a significant increase of tubular volumes in GN rats compared with control rats. Treatment with candesartan and candesartan/pirfenidone reduced tubular enlargement significantly. Combination treatment could not significantly improve the effect of candesartan treatment alone. The decrease...
of tubular volumes achieved in the pirfenidone-treated group was not significantly different from that of GN rats. There was no significant increase of interstitial and post-glomerular capillary volumes in GN rats compared with control rats. Therefore, the effect of either therapy could not be proven referring to these parameters.

Representative electron micrographs of glomerular changes are shown in Figure 5. There was marked podocyte pathology in GN rats. Podocytes were enlarged and displayed focal foot process fusion with a prominent cytoskeleton along the basement membrane. Many podocytes contained numerous large

**Ultrastructural findings**

Representative electron micrographs of glomerular changes are shown in Figure 5. There was marked podocyte pathology in GN rats. Podocytes were enlarged and displayed focal foot process fusion with a prominent cytoskeleton along the basement membrane. Many podocytes contained numerous large
adsorption droplets and lots of pseudocysts were seen. Particularly in areas with segmental sclerosis, electron-dense deposits representing proteinaceous material were observed. The basement membrane showed irregularities with uneven thickness and basement membrane protrusions.

Few electron-dense deposits were seen in rats treated with either candesartan or pirfenidone, but still basement membrane irregularities could be easily detected and podocytes covering such irregular segments exhibited a prominent cytoskeleton and focal foot process fusion. Rats treated with a combination of candesartan and pirfenidone revealed nearly normal morphology in ultrastructure and basement membrane alterations, and podocyte damage could be hardly detected at all.

**Collagen Iα mRNA expression**

Collagen Iα mRNA expression was significantly enhanced in GN rats compared with control rats (Figure 6). As with all other clinical and morphological parameters collagen Iα expression was clearly reduced to nearly normal levels in all treated groups. This result corresponds to minor interstitial fibrosis observed in histological sections of the rats treated with candesartan, pirfenidone or combination treatment. There were no significant differences in collagen Iα mRNA expression between treatment groups. However, there was a tendency to somewhat greater reduction of collagen Iα mRNA by candesartan compared with pirfenidone.

**Immunohistochemistry**

The glomeruli showed a significantly increased number of ED1- (2.61 ± 0.25/glomerulus, *P* < 0.001 vs control)
and W3/13-positive cells (3.69 ± 0.44/glomerulus, P < 0.001 vs control) in the GN group compared with the control group (0.18 ± 0.03 ED1-positive cells/glomerulus, 0.92 ± 0.09 W3/13-positive cells/glomerulus; Figure 7). Surprisingly, there were even higher counts of ED1- and W3/13-positive glomerular cells in all treatment groups compared with the GN group. The number of ED1-positive glomerular cells in the pirfenidone-treated group (3.99 ± 0.47 ED1-positive cells/glomerulus) and the number of W3/13-positive glomerular cells in the candesartan-treated group (5.93 ± 0.74 W3/13-positive cells/glomerulus) were significantly higher than in the GN group (P < 0.05).

The number of ED1-positive cells in the interstitium was increased in the GN group (7.51 ± 1.68 positive cells/HPF) compared with the control group (1.81 ± 0.18 positive cells/HPF; P < 0.01). Treatment reduced the number of ED1-positive interstitial cells...
to $4.36 \pm 0.68$ positive cells/HPF in the candesartan-treated group ($P = 0.14$ vs GN) and to $4.79 \pm 1.31$ positive cells/HPF in the pirfenidone-treated group ($P < 0.05$ vs GN group). The number of ED1-positive cells in the group treated with both drugs was $2.98 \pm 0.30$ ED1-positive cells/HPF ($P = 0.14$ vs the candesartan group).

The number of W3/13-positive cells in the interstitium was slightly, but not significantly, raised in the GN group, $(7.44 \pm 1.74$ positive cells/HPF) compared with the control group $(4.19 \pm 0.31$ positive cells/HPF). There was only a numerical, but no significant, difference of the number of W3/13-positive interstitial cells in the groups treated with candesartan $(5.98 \pm 0.39$ positive cells/HPF) and, pirfenidone $(7.09 \pm 1.05$ positive cells/HPF) compared with the GN group. The number of W3/13-positive interstitial cells in the group with combination therapy $(5.47 \pm 0.23$ positive cells/HPF) was also not different from the other groups.

**Fig. 6.** Effect of treatment on cortical collagen I α mRNA expression. GN = glomerulonephritis group; Cd = candesartan group; Pfd = pirfenidone group; Cd/Pfd = group with combination treatment. Values are shown as mean±SE; *$P < 0.01$ vs GN (ANOVA).

**Fig. 7.** Immunohistochemical investigations: ED1-positive cells in glomeruli (a) and interstitium (c); W3/13-positive cells in glomeruli (b) and interstitium (d); semi-quantitative score of nitrotyrosine positivity (e). GN = glomerulonephritis group; Cd = candesartan group; Pfd = pirfenidone group; Cd/Pfd = group with combination treatment. Values are shown as mean±SE; *$P < 0.05$ vs GN, #$P < 0.05$ vs Cd (Mann–Whitney tests).
Nitrotyrosine positivity was seen in tubular epithelial cells and in endothelial cells of few arteries and veins. The semi-quantitative score of tubular positivity was increased in the GN group (3.00 ± 0.37) compared with the control group (1.75 ± 0.31; P < 0.05). The groups treated with candesartan (2.50 ± 0.31) and pirfenidone (2.33 ± 0.44) had numerical but not significantly lower values than the GN group (P = 0.56 candesartan vs GN, P = 0.24 pirfenidone vs GN). The group with combined treatment showed a score of 1.33 ± 0.17, which was significantly different compared with the candesartan group (P < 0.05) (Figure 8).

**Discussion**

The main information that emerges from the present study is that pirfenidone has a beneficial effect on functional and morphological parameters of glomerular and tubulointerstitial damage in longstanding GN. Proteinuria, podocyte damage, tubular degeneration and collagen Iα expression are significantly reduced compared with rats with untreated GN. Reduction of the above-mentioned parameters is comparable with that produced by candesartan, and emphasizes the renoprotective potential of pirfenidone which extends beyond its antifibrotic properties. The percentage of glomeruli with adsorption droplets is significantly reduced by combination treatment compared with candesartan monotherapy. This finding indicates a potential additive effect of combined treatment. Our results are new and have, to the best of our knowledge, not been published before. The renoprotective effect of candesartan is well known, but there are only few reports using this type of GN (anti-GBM GN).

The effects of pirfenidone in GN have been studied in anti-Thy-1 GN by Shimizu *et al.* [6]. In spite of different mechanisms during induction and maintenance of kidney disease, the rats developed a similar pattern of renal pathology to that observed in our study. After 6 weeks, segmental sclerosis was found in ~20% of glomeruli and was associated with tubulointerstitial damage [12]. Pirfenidone ameliorated proteinuria, glomerular sclerosis and tubulointerstitial damage. Our study confirms the beneficial effect of pirfenidone but expands the information from this previous report in several ways. First, our study proved the effect of pirfenidone on chronic tubulointerstitial damage in GN by quantification of tubular degeneration and collagen Iα expression. Secondly, we demonstrated at the ultrastructural level that pirfenidone might affect damaging processes in the glomerular filtration barrier in general.

Podocyte damage is thought to be an important prerequisite for development of focal and segmental sclerosis in anti-GBM GN [13]. Foot process fusion, a prominent cytoskeleton and adsorption droplets are regarded as potentially reversible podocyte lesions. Adsorption droplets in podocytes stained PAS positive and could be easily detected using light microscopy under high magnification. There was a good correlation (correlation coefficient 0.873) between the percentage of glomeruli with adsorption droplets in podocytes and the amount of proteinuria. Adsorption droplets in podocytes are, like proteinuria, an indicator of distortion of the filtration barrier [14]. Pirfenidone significantly reduced the number of glomeruli with
glomerulosclerosis, interstitial fibrosis and expression to the same levels as the control group and reduced interstitial changes. Candesartan decreased proteinuria of long-lasting GN with respect to the effect of glomerular lesions such as adherences and segmental nidone might prevent development of irreversible damage and consecutive reduction of segmental sclerosis.

Few investigations have been carried out in rat models of long-lasting GN with respect to the effect of candesartan on irreversible glomerular and tubulo-interstitial changes. Candesartan decreased proteinuria to the same levels as the control group and reduced glomerulosclerosis, interstitial fibrosis and expression of TGF-β, collagen I and III in chronic anti-Thy-1 GN in rats over a time period of 10 weeks [15]. We are able to confirm the beneficial effect of candesartan in anti-GBM GN.

In comparison with pirfenidone, candesartan seems to have a slightly better effect in the present study of anti-GBM GN. Candesartan treatment resulted in greater reduction of proteinuria as well as the percentage of glomeruli with segmental sclerosis and adsorption droplets in podocytes, tubular degeneration, interstitial fibrosis and collagen Iα mRNA expression. Differences between the candesartan- and pirfenidone-treated groups were not statistically significant; the consistent pattern nevertheless suggests a superior renoprotective effect of candesartan treatment.

Even if there are slight quantitative differences, the qualitative effects on glomerular and tubular morphology are strikingly similar. Improvement of podocyte damage and consecutive reduction of segmental sclerosis is probably not due to a reduction of glomerular inflammatory cells, as the number of glomerular ED1-positive macrophages and CD43-positive cells was even higher in the treatment groups compared with the GN group. A similar phenomenon was reported by Zhou et al. in anti-GBM GN [16]: crescent formation and interstitial fibrosis were reduced by blockade of TGF-β with its soluble type II receptor expressed in an adenovirus vector; however, the number of glomerular ED1-positive cells was increased in the treatment group at day 14. They related this finding to an increased glomerular expression of monocye chemotactic protein-1 (MCP-1). It is conceivable that the increased number of glomerular macrophages in our treatment groups might be an effect mediated by TGF-β, as both candesartan and pirfenidone are reported to reduce expression of TGF-β [5,15].

As proteinuria, podocyte damage and segmental sclerosis are clearly reduced in the treatment groups, the increased number of glomerular inflammatory cells could not be related to increased tissue damage. The cells might be involved in repair processes such as removal of deposited immune complexes and fibrin.

The first study of pirfenidone [2] emphasized a possible interference of the drug with the generation of oxygen radicals. Pirfenidone diminished increased lipid peroxidation and increased the levels of superoxide dismutase. Another study demonstrated that pirfenidone also inhibited NADPH-dependent lipid peroxidation, and that pirfenidone was a scavenger for hydroxyl radicals [17]. Reactive oxygen species have been proved to play a role in the early phase of anti-GBM GN in rats [18]. Nitrotyrosine is an end product of peroxynitrite formation by nitric oxide and superoxide. In the present study, it could not be detected in glomeruli at all, neither in the GN group nor in the treatment groups, and an effect of pirfenidone and candesartan on generation of reactive oxidative species in glomeruli could not be proved in this stage of the disease using this method.

The renoprotective effect of inhibitors of the renin-angiotensin system is at least in part attributed to their ability to normalize glomerular capillary pressure by dilation of the efferent arteriole. Systemic blood pressure clearly decreased under candesartan therapy, and plasma renin levels were strongly increased as a consequence of AT1 receptor blockade. The glomerular intracapillary pressure is enhanced in the autologous phase of anti-GBM GN and the glomerular filtration rate is maintained with a reduced glomerular capillary ultrafiltration coefficient [19]. This may lead to increased wall tension and capillary dilation with increased mechanical stress on the podocytes [14]. Even if the initial injury in anti-GBM GN is of an immunological nature, alteration of glomerular haemodynamics by candesartan is shown to attenuate proteinuria and preserve glomerular architecture as demonstrated by a decrease in podocyte damage and segmental glomerulosclerosis.

Rats treated with pirfenidone had the same blood pressure as control rats, and plasma renin levels were decreased to the same extent as in the GN group. Thus pirfenidone seems to have no effect on the renin-angiotensin system, and a beneficial effect via alteration of glomerular haemodynamics seems unlikely.

Combined treatment resulted in the lowest proteinuria levels during the experiment and the best preserved morphology at the end of the study demonstrated by the lowest values of indicators of glomerular and tubulointerstitial damage. The numbers of glomeruli with adsorption droplets in podocytes and the grade of nitrotyrosine positivity in tubules were significantly reduced by combined treatment compared with candesartan treatment. This indicates that pirfenidone might improve renoprotection achieved by candesartan. Segmental glomerular sclerosis, tubular degeneration and interstitial fibrosis were reduced to a greater extent by combined treatment; however, the differences were only numerically but not significantly different compared with candesartan treatment.

The combination group showed the lowest values for blood pressure and body weight during the study. Blood pressure was significantly reduced after 3 weeks by combination treatment even in comparison with candesartan treatment. However, the combination group had significantly lower body weights than all other groups. We did not measure the amount of daily...
food intake, but it is likely that it was lower in the group treated with both drugs. Both hypotension and low protein diet have an independent beneficial effect on proteinuria and glomerular morphology [20]. We cannot exclude that the favourable results in the group treated with both drugs might, at least in part, be due to very low blood pressure values and possibly also low protein intake, but this part of the study needs further investigation.

In conclusion, both pirfenidone and candesartan were found to have beneficial effects on proteinuria and morphological changes in chronic anti-GBM GN. There was a trend towards slightly better preserved function and morphology with candesartan treatment compared with pirfenidone treatment. Combined treatment with candesartan and pirfenidone resulted in superior preservation of morphology. Pirfenidone, given either alone or in combination with other drugs, might therefore offer a new therapeutic tool in the treatment of chronic GN in man.

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