Combination Therapy with Telmisartan and Oxacalcitriol Suppresses the Progression of Murine Adriamycin Nephropathy

Running title: Telmisartan and oxacalcitriol in ADR-nephropathy

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Key words Adriamycin nephropathy· AT1 receptor antagonist· Podocyte· Vitamin D analog
Abstract

Background: Blockade of the renin-angiotensin system plays a key role in suppressing the progression of renal diseases. It has not been well established whether this therapy provides additional effects when combined with vitamin D or its analog in an Adriamycin (ADR)-induced nephropathy model.

Methods: We evaluated the effect of an angiotensin II subtype 1 (AT₁) receptor blocker (telmisartan) combined with a vitamin D analog (oxacalcitriol) on mice ADR-induced nephropathy (9.5mg/kg single intravenous injection). We also tested immortalized murine podocytes to examine the effects on podocyte apoptosis.

Results: Mice with ADR-induced nephropathy developed progressive albuminuria and glomerulosclerosis within 30 days accompanied by decreased expression of slit diaphragm (SD)-associated proteins (nephrin and podocin), reduced numbers of podocytes, and increased systolic blood pressure. Treatment with telmisartan or oxacalcitriol alone moderately ameliorated kidney injury. The combined treatment most effectively reduced the albuminuria and glomerulosclerosis. These effects were accompanied by the restoration of SD-associated proteins, reduction of podocyte apoptosis, and prevention of podocyte depletion in the glomeruli. Treatment with telmisartan, oxacalcitriol, and the combination therapy resulted in similar reductions in systolic blood pressure. In cultured murine podocytes, ADR stimulated the expression of Bax/Bcl-2 and apoptosis as determined by Hoechst 33342 staining. These changes were effectively inhibited by telmisartan or oxacalcitriol, but the combination treatment most effectively reduced these effects.

Conclusions: These data demonstrated that application of a renin-angiotensin system blocker plus a vitamin D analog effectively prevented renal injury in ADR-induced nephropathy. The observed amelioration of renal injury may be partly attributable to anti-apoptotic effects in podocytes.
Introduction

Focal segmental glomerulosclerosis (FSGS), which progresses to end-stage kidney disease, is the leading cause of nephrotic syndrome in adults. The murine model of adriamycin (ADR)-induced nephropathy, an experimental model of FSGS in humans, has demonstrated that BALB/c mice exposed to ADR developed podocyte injury, which resulted in severe proteinuria and progressive renal failure [1,2]. Podocytes are highly specialized, terminally differentiated epithelial cells that play a central role in the development of proteinuria and various kidney diseases [3]. Furthermore, podocyte apoptosis is a critical process in the establishment of FSGS, and this ADR-induced nephropathy model is ideal for clarifying the underlying mechanisms of the response of podocytes to injury in chronic kidney disease (CKD) [4].

The renin-angiotensin system (RAS) plays a key role in the development of renal diseases. Blockade of the RAS is widely used to suppress progression of experimentally-induced renal diseases. The ability of RAS blockade to influence podocyte injury has been previously demonstrated in early diabetic nephropathy [5]. Moreover, inhibiting the RAS was shown to prevent the rearrangement of slit diaphragm (SD)-proteins, including nephrin, and to prevent progressive renal damage in experimental animals with proteinuric nephropathy [6]. Suzuki et al. also demonstrated that the angiotensin II subtype 1 (AT1) receptor mediated a reduction in the expression of podocyte functional molecules [7].

It has been reported that calcitriol (a form of vitamin D) and its analog, 22-oxa-calcitriol (oxacalcitriol), had therapeutic potential for ameliorating the progression of glomerulosclerosis [8-10]. We recently used oxacalcitriol to treat mice with ADR-induced nephropathy, and we demonstrated that oxacalcitriol ameliorated glomerular injury through its podocyte-protective activity [11]. Zhang et al. reported that inhibiting the RAS with a combination of vitamin D analogs had a synergistic therapeutic effect in diabetic renal disease [12,13]. Some evidence has suggested that the combination of an AT1 receptor blocker (ARB) and a vitamin D analog resulted in superior therapeutic efficacy compared to mono-therapy in experimental renal disease [12,13]. Although a multimodal treatment strategy to protect the kidney has been introduced in patients with CKD [14], it remains to be
determined whether a combination of an ARB and a vitamin D analog could exert therapeutic effects on the intervention or prevention of ADR-induced nephropathy.

In the present investigation, our specific objectives were to investigate the effects of applying the combination of ARB and a vitamin D analog for treating ADR-induced nephropathy and to evaluate whether the observed effects were dependent on ameliorating podocyte injury.
Methods

Animals, drug treatment, and sample collection

Female BALB/c mice weighing 20-25g and aged eight weeks were purchased from an animal care facility. Nephrotic syndrome was induced via a single tail-vein injection of 9.5mg/kg ADR (doxorubicin hydrochloride; Wako Chemicals, Japan), while the same volume of isotonic saline was injected into age-matched normal control mice. After ADR injection, the mice were randomly separated into four groups and treated with vehicle, with 0.05μg/kg oxacalcitriol (Chungai Pharmaceutical, Japan; dissolved in phosphate-buffered saline containing 0.2% ethanol and 0.01% Tween20, i.p. injection three times per week), with 0.1mg/kg telmisartan (Nippon Boehringer Ingelheim, Japan; dissolved in phosphate-buffered saline, i.p. injection everyday), or a combination of both drugs. Three mice from each group were sacrificed on day3, day5 and day 15 after ADR injection, and five mice were sacrificed on day30 after ADR injection.

For the isolation of glomeruli, another 12 mice were injected with ADR, grouped using the method described above, and sacrificed on day 15. The glomeruli were isolated via the sieving method and used for the purification of protein. Spot urine was collected, and urinary albumin and creatinine levels were determined by enzymatic assays using commercial kits (DCA200 system, Siemens, Germany). Systolic blood pressure (SBP) was measured in conscious mice using the automated tail-cuff method. Procedures for the present study were approved by the Animal Committee at Juntendo University Faculty of Medicine, and all animals were treated according to the guidelines for animal experimentation at Juntendo University, Tokyo, Japan.

Podocyte culture

Conditionally immortalized mouse podocytes were cultured as described previously[15]. Differentiated podocytes were treated with 0.25μg/mL ADR in regular RPMI medium for 20h. To explore the effect of drugs, podocytes were pretreated with 10⁻⁸M oxacalcitriol for 3 h and 10⁻⁷M telmisartan for 1h.
**Analysis of renal morphology**

Light microscopy was performed according to methods described previously [16]. In periodic acid-Schiff-stained sections, at least 30 midsections of glomeruli were used for this study. Glomerular and Bowman’s capsule areas were carefully traced by hand and measured using a digitizer KS-400 Imaging System; the ratio of glomerular to Bowman’s capsule (G/B) volume was calculated as described previously [17]. One hundred randomly selected, full-sized glomeruli from each mouse were used for the semi-quantitative evaluation of glomerular lesions. The sections were analyzed in a blind manner, and the rate of sclerotic glomerular formation (sclerosis>50% of the glomerulus) was determined.

**Primary antibodies**

Polyclonal guinea pig anti-nephrin antiserum (Progen, Germany), polyclonal rabbit anti-WT1 antiserum (Santa Cruz Biotechnology, USA), monoclonal mouse anti-WT1 antibody (Dako Corporation, USA), monoclonal mouse anti-synaptopodin antibody (Progen), polyclonal rabbit anti-Bax antiserum (Cell Signaling Technology, USA), polyclonal anti-Bcl-2 antiserum (Cell Signaling Technology, USA), and monoclonal mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Abcam, USA) were purchased for immunofluorescence (IF) and immunoblotting. Polyclonal rabbit anti-podocin antiserum has been described previously [18].

**Immunofluorescence**

Slides containing sections were incubated with primary antisera (guinea pig anti-nephrin antibody and rabbit anti-podocin antibody, 1:50 dilution) at room temperature, washed in phosphate-buffered saline, and incubated with the specific secondary antibody. For semi-quantitative analysis, the extent of glomerular expression was evaluated for at least 50 glomeruli by two blind observers. Scores were assigned to individual glomeruli from each section as follows: a score of 4, the area in which continuous staining was strikingly disrupted and the staining intensity was clearly decreased...
covered 0-25% of the glomerular tuft area; a score of 3, 26-50%; a score of 2, 51-75%; and a score of 1, 76-100%. The final score for each mouse was then calculated as described previously[11].

**Immunoblotting**

Immunoblotting of isolated glomerular extracts was performed as described previously[19]. Polyclonal antibodies against podocin and nephrin were diluted 1:500. The density of the positive bands was quantitated using imaging analysis with LAS-3000 (Fujifilm, Japan). This procedure was carried out three times. The ratio of the densitometric signal of the molecules was compared with that of GAPDH. Data are shown as ratios relative to control and are expressed as means ± standard error (SE) of the results of three independent experiments. Podocytes were harvested from plates and evaluated for the expression of Bax and Bcl-2, as described previously [20].

**TUNEL assay and WT1 immunofluorescence**

Apoptosis was identified within glomeruli via a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay using a commercially available kit (Chemicon, USA). The number of TUNEL-positive glomerular cells in paraform aldehyde-fixed renal tissue was determined by examining at least 100 glomeruli at 400x magnification. Glomerular podocytes were identified with WT1 as a podocyte-nucleus marker. To determine the number of podocytes in glomeruli, 30 glomeruli in each section were randomly examined on day 30.

**Hoechst 33342 staining and podocyte apoptosis marker analysis**

Podocyte apoptosis was identified in cultured podocytes seeded on cover slips and stained with Hoechst 33342 (Molecular Probes, USA). Hoechst 33342 was added at a final concentration of 10 μM and the cells were analyzed after 5 min. Apoptotic cells were defined by the presence of nuclear condensation. The percentage of podocytes with nuclear condensation was determined in a minimum of 200 cells.
Statistical analysis

Data are presented as means ± SE. Statistical comparisons were made via analysis of variance, with $P<0.05$ considered significant.
Results

Physical and biochemical parameters

Mice with ADR-induced nephropathy displayed a significant decrease in body weight on day 30 after all treatment regimens (Table 1). There were no significant changes in the levels of serum creatinine or calcium in all groups. Oxacalcitriol and the combination drug treatment exerted significant inhibitory effects on the development of albuminuria from day 5 (fig. 1a). Treatment with oxacalcitriol or telmisartan alone similarly attenuated the progression of albuminuria in mice with ADR-induced nephropathy on day 30 (vehicle: 384.50±34.85 µg/mg Cr; oxacalcitriol: 251.30±9.20 µg/mg Cr; telmisartan: 244.20±13.60 µg/mg Cr). Moreover, the combination drug treatment yielded the greatest inhibitory effects on albuminuria (104.00±5.84 µg/mg Cr). On day 30, mice with ADR-induced nephropathy had higher systolic blood pressure (SBP) than control mice (121.20±2.05 mmHg vs. 100.30±0.33 mmHg). Treatments with oxacalcitriol, telmisartan, and the combination similarly reduced in SBP (108.00±1.04 mmHg, 105.00±1.70 mmHg, and 104.00±1.58 mmHg, respectively, on day 30; fig. 1b).

Histological findings

On day 30, ADR-treated mice exhibited global glomerulosclerosis, tubular collapse, and severe interstitial expansion in periodic acid-Schiff staining (fig. 1c). The glomerulosclerosis formation rate (fig. 1d) in kidney sections was highest in vehicle-treated mice; significant reductions in this rate were observed in mice treated with oxacalcitriol, telmisartan, or the combination (17.33±0.33%, 9.33±1.20%, 12.67±0.88%, and 5.33±1.52%, respectively, on day 30). Mice treated with the combination showed a significant reduction in the number of sclerotic glomeruli compared to mice treated with oxacalcitriol or telmisartan alone.

To evaluate glomerular hypertrophy, we calculated the G/B ratio on day 30. In the vehicle-treated group, the G/B ratio was significantly increased compared to that of the control group. The G/B ratio of combination-treated mice remained the same as that of the control group (fig. 1e).

Expression of SD-associated proteins in glomeruli: immunofluorescence and immunoblotting
In glomeruli of normal mice, nephrin and podocin were stained in a continuous linear pattern along the glomerular capillary loop. In ADR-treated mice, staining intensities were reduced and disrupted on day 5, and the greatest decrease in glomeruli was observed on day 15. Then, the staining patterns had partially recovered on day 30. In the drug treatment groups, the intensities of nephrin and podocin were significantly stronger and less disrupted than those in vehicle-treated glomeruli. Furthermore, the staining intensities in the combination treatment group were strongest among the groups on day 15 (fig. 2a). In semi-quantitative analyses, SD-associated protein expression was scored on a scale of 1 to 4 on days 5 and 15 (fig. 2b). On day 5, treatment with oxacalcitriol, telmisartan, or the combination resulted in similar attenuations in the SD-associated protein score. On day 15, the combination treatment was associated with the best attenuating effects on SD-associated proteins among the treatment groups.

The abundance of nephrin and podocin proteins on day 15 of ADR-induced nephropathy was analyzed in glomerular lysates with immunoblotting (fig. 2c). ADR-induced nephropathy was associated with a clear reduction in the abundance of nephrin and podocin. Treatment with oxacalcitriol or telmisartan slightly increased the amounts of these proteins compared to levels observed in the vehicle-treated group. However, the combination treatment resulted in greater protein expression in the glomeruli compared to the other treatment groups (fig. 2d).

Podocyte loss and apoptosis in mice with ADR-induced nephropathy: TUNEL assay and WT1 staining

Podocyte apoptosis is a major cause of podocyte loss. Podocyte loss leads to proteinuria and glomerulosclerosis. The largest number of apoptotic cells was detected on day 3 after ADR injection in a previous study [4]. Therefore, we assessed podocyte apoptosis on day 3 with TUNEL staining (fig. 3a). We found no apoptotic cells in kidney sections of animals in the control group. Similar to previous studies, apoptotic cells were noted in the glomeruli of mice with ADR-induced nephropathy (0.81±0.16%) [4,11]. In contrast, among mice with ADR-induced nephropathy, those that received combination treatments had the smallest proportion of apoptotic cells (0.30±0.26%, fig. 3b). In our previous study, podocyte loss was detected from day 15, and the number of podocytes was
significantly decreased on day 28 after ADR injection [4]. Therefore, on day 30, we counted podocytes after staining the nuclei with WT1 and counterstaining with synaptopodin (fig. 3c). Mice with ADR-induced nephropathy showed significantly decreased numbers of WT1-positive nuclei in the glomeruli. Mice treated with oxacalcitriol and telmisartan showed higher numbers of nuclei in the glomeruli, but those treated with the combination showed nearly normal numbers of nuclei in the glomeruli (fig. 3d).

*Apoptosis-related molecules and apoptosis in cultured podocytes: Hoechst 33342 staining and immunoblotting*

To examine the effects of ADR and/or treatment drugs on apoptosis-related molecules, we investigated the expression of Bax and Bcl-2 in podocyte (fig. 4a,b). Adriamycin significantly induced Bax and significantly reduced Bcl-2 protein expression in cultured podocytes. These changes were effectively inhibited by treatment with oxacalcitriol or telmisartan. Even greater efficacy was achieved with the combination treatment, which resulted in the lowest Bax/Bcl-2 ratio. The number of apoptotic cells, assessed with Hoechst 33342 staining, was significantly increased in ADR-stimulated cells; this increase was ameliorated by treatment with oxacalcitriol or telmisartan (vehicle, oxacalcitriol, and telmisartan: 38.97±1.66 %, 23.33±2.02 %, and 25.90±1.05 %, respectively). Combination-treated cells exhibited a greater reduction in apoptosis (15.40±1.22%) compared to cells treated with telmisartan or oxacalcitriol alone (fig. 4c,d).
Discussion

In this study, we demonstrated that a therapy comprising a combination ARB and a vitamin D analog nearly completely prevented the progression of albuminuria and glomerulosclerosis in the ADR-induced nephropathy model. Previous studies have focused on multidrug strategies that included combining RAS inhibitors with other drugs for protecting the kidney in renal disease. Another previous study showed that a combination of an ARB and a vitamin D analog produced therapeutic synergism in the prevention of diabetic nephropathy [12,13]. Zhang \textit{et al.} also demonstrated that the therapeutic effects of vitamin D analogs were improved by combining them with RAS inhibitors to suppress the compensatory renin increase [12].

We also showed that treatment with the ARB telmisartan yielded renoprotective effects by ameliorating podocyte injury in ADR-induced nephropathy. Similar results were demonstrated by Ibrahim \textit{et al.}[21], who noted significantly attenuated renal injury following telmisartan treatment in ADR-exposed mice. However, Tang \textit{et al.}[2] did not find similar effects with losartan. Those conflicting results were probably related to drug-specific effects. Recent studies have shown that telmisartan modulated peroxisome proliferator-activated receptor gamma (PPAR-\(\gamma\)) [22,23]. Telmisartan showed renoprotective effects through the PPAR-\(\gamma\) pathway, independent of AT\(_1\) receptor blockade [24]. Also, a previous study reported that telmisartan reduced apoptosis via the PPAR-\(\gamma\) pathway [25]. PPAR-\(\gamma\) is normally expressed in podocytes, and its activation is protective against podocyte apoptosis [26]. Zhou \textit{et al.}[27] also reported that treatment with a PPAR-\(\gamma\) agonist decreased proteinuria and attenuated glomerulosclerosis in the ADR-induced nephropathy model. It has been suggested that the PPAR-\(\gamma\) agonist-induced renoprotective effect was dependent, at least partially, on the protection against podocyte apoptosis and the regulation of nephrin expression [28].

As reported previously[2], this study demonstrated that mice with ADR-induced nephropathy had elevated SBP. The antihypertensive effect of a vitamin D analog was shown in another previous study, and the mechanism was postulated to be related to reducing the activity of the RAS [29]. However, the combination of telmisartan and oxacalcitriol did not yield any benefits beyond those achieved with oxacalcitriol treatment alone; the antiproteinuric effect of an ARB and a vitamin D analog in ADR-treated mice has been shown to be dependent on both the antihypertensive and the anti-apoptotic
effects on podocytes. In addition, this result may be related to our use of each drug at sufficiently effective doses; thus, the effects of adding another agent were obviated, and the SBP was not reduced further.

Nephrin and podocin play important roles in the formation of the SD. Thus, disturbances in the expression of these proteins result in proteinuric renal diseases, including FSGS and diabetic nephropathy [30]. In previous studies, angiotensin II antagonist treatment abrogated the downregulation of nephrin in the diabetic model, and vitamin D treatment reduced podocyte apoptosis and nephrin loss [31,32]. At the molecular level, our data showed that the observed renoprotective effects were accompanied by an attenuation of the ADR-induced alteration of nephrin and podocin in the glomeruli. At the molecular level, our data showed that the observed renoprotective effects were accompanied by attenuation of the ADR-induced alterations in nephrin and podocin in the glomeruli. Although the antiproteinuric effect of oxacalcitriol was stronger than that of telmisartan on day 5, both drugs similarly attenuated the alterations in SD-protein expression. The mechanisms underlying proteinuria in response to podocyte injury are multiple and complex. Proteinuria develops from alterations in the SD proteins, reduced podocyte numbers, podocyte effacements, and glomerular endothelial cell dysfunction [33]. In this study, we evaluated only SD protein expression and podocyte numbers. Further study is needed to explain the discrepancy between the level of proteinuria and the level of SD-protein expression on day 5.

It is known that a reduction in podocyte number is closely associated with the development of proteinuria and glomerulosclerosis in renal diseases[34]. Podocyte apoptosis is a key step in the development of podocyte loss. Also, this process is thought to be associated with the reversibility of glomerulosclerosis [35]. Our previous study demonstrated that the largest degree of apoptosis occurred on day 3, and the greatest podocyte loss occurred on day 28 after ADR injection [4]. Consequently, it was thought that podocyte apoptosis was characteristic of the early stages of ADR-induced nephropathy, and podocyte loss or glomerular sclerosis was a landmark of late stage ADR-induced nephropathy. A recent study demonstrated that proteinuria increased as podocyte number decreased [36]. In this study, the combination therapy provided the best protection against podocyte
loss on day 30. Consistent with pathological findings, the present study showed that the combination drug yielded the best inhibition of albuminuria on day 30.

The present *in vitro* analyses showed that ADR induced both upregulation of the pro-apoptotic molecule, Bax, and downregulation of the anti-apoptotic molecule, Bcl-2. These alterations were effectively inhibited by treatment with oxacalcitriol or telmisartan; moreover, the combination therapy showed the greatest inhibitory effects. Our results suggested that the upregulation of Bcl-2 and downregulation of Bax in podocytes may represent the protective effects of oxacalcitriol and telmisartan treatment in injured podocyte lesions; thus, this dual regulation may have limited podocyte apoptosis.

This study had some limitations. First, we did not demonstrate the exact mechanisms of telmisartan and/or oxacalcitriol on podocyte loss and apoptosis in ADR-induced nephropathy. Further studies are required to clarify the potential mechanism; for example, the effects of telmisartan on the PPAR-γ pathway. Furthermore, it is necessary to evaluate the specific mechanisms underlying the synergistic effects of combining an ARB and a vitamin D analog. To demonstrate the drug’s therapeutic effect more clearly, a future study might examine the effects of applying telmisartan and oxacalcitriol after establishing proteinuria in ADR-treated mice.

In conclusion, the results of this study indicated that glomerular podocytes exposed to ADR underwent apoptosis and lost functional molecules, such as nephrin and podocin. These deteriorations were ameliorated by the combination of telmisartan and a vitamin D analog through their antiapoptotic effects on podocytes in ADR-induced nephropathy.
Acknowledgment

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Disclosure Statement

The authors declare no conflict of interest.
**Figure Legends**

**Fig. 1.** Effects of drug treatment on the development of albuminuria, systolic blood pressure, and pathological findings.

(a) Treatment with oxacalcitriol or telmisartan similarly attenuated the progression of albuminuria in ADR-induced nephropathy. The combination drug treatment yielded the greatest inhibitory effects on albuminuria

(b) Treatments with oxacalcitriol, telmisartan, and the combination similarly reduced n SBP

(c) Representative glomerular morphology of control mice and mice with ADR-induced nephropathy treated with vehicle, oxacalcitriol, telmisartan, or the combination therapy. (d) The glomerulosclerosis formation rate was highest in the vehicle-treated mice. Mice treated with the combination showed a significant reduction in the number of sclerotic glomeruli compared to mice treated with oxacalcitriol or telmisartan alone. (e) The G/B ratio of combination-treated mice remained the same as that of the control group. Error bars indicate SE. *P<0.05 vs. control; #P<0.05 vs. vehicle; **P<0.05 vs. vehicle and telmisartan; ***P<0.05 vs. mice treated with vehicle, or with oxacalcitriol or telmisartan alone.

**Fig. 2.** Expressions of slit diaphragm (SD)-associated proteins on days 5, 15 and 30.

(a) Representative expression of SD-associated proteins, as assessed by IF. In ADR-treated mice, staining intensities were reduced and disrupted on day 5, and the greatest decrease in glomeruli was observed on day 15. The staining intensities in the combination treatment group were strongest among the treatment groups on day 15. (b) Semi-quantitative IF scores on day 5 and 15. (c) Immunoblotting against SD-associated proteins in the glomerular lysate on day 15. (d) Semi-quantitative analysis of immunoblotting. Treatment with oxacalcitriol or telmisartan slightly increased the amounts of SD-associated proteins in this group, as compared to the vehicle-treated group. In addition, the combination treatment resulted in an increase in protein expression in glomeruli vs. other treatment groups. Error bars indicate SE.*P<0.05 vs. control; #P<0.05 vs. vehicle; ***P<0.05 vs. mice treated with vehicle, or with oxacalcitriol or telmisartan alone.
Fig. 3. Podocyte loss and apoptosis markers.

(a) A representative TUNEL-positive cell in the glomerulus of an ADR-exposed mouse on day 3. (b) Compared with mice with ADR-induced nephropathy that did not receive treatment, combination-treated mice had fewer apoptotic cells. (c) IF staining against WT1 (green) and synaptopodin (red) on day 30. (d) Mean podocyte number/glomerulus is shown for at least 30 glomeruli in each group. Mice with ADR-induced nephropathy harbored significantly fewer WT1-positive cells, while this number increased in oxacalcitriol- and telmisartan-treated glomeruli and was highest in the glomeruli of combination-treated mice. Error bars indicate SE. *P<0.05 vs. control; #P<0.05 vs. vehicle; ***P<0.05 vs. mice treated with vehicle, or with oxacalcitriol or telmisartan alone.

Fig. 4. Apoptosis-related molecules and apoptosis in immortalized mouse podocytes.

(a) Representative immunoblot of Bax and Bcl-2 in immortalized mouse podocytes. (b) Adriamycin significantly induced Bax and significantly reduced Bcl-2 protein expression in cultured podocytes. These changes were effectively inhibited by treatment with oxacalcitriol or telmisartan. The combination treatment was even more effective, resulting in the lowest Bax/Bcl-2 expression. (c) Representative Hoechst 33342 staining. (d) An increase in the number of apoptotic cells in ADR-treated cells was ameliorated by treatment with oxacalcitriol or telmisartan. Combination-treated cells showed more reduction of apoptosis than cells treated with telmisartan or oxacalcitriol alone. Error bars indicate SE. *P<0.05 vs. control; #P<0.05 vs. vehicle; ***P<0.05 vs. vehicle and vs. cells treated with oxacalcitriol and telmisartan alone.
References


Telmisartan and Oxacalcitriol in ADR-Nephropathy


Table 1. Physical and biochemical parameters at day 30

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vehicle</th>
<th>Oxacalcitriol</th>
<th>Telmisartan</th>
<th>Combination</th>
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</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>24.20±0.17</td>
<td>19.72±0.66*</td>
<td>21.28±0.41*</td>
<td>19.70±0.28*</td>
<td>20.95±0.78*</td>
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<tr>
<td><strong>SCr (mg/dL)</strong></td>
<td>0.10±0.0</td>
<td>0.20±0.10</td>
<td>0.10±0.0</td>
<td>0.14±0.02</td>
<td>0.10±0.0</td>
</tr>
<tr>
<td><strong>SCa²⁺ (mg/dL)</strong></td>
<td>8.83±0.50</td>
<td>8.42±0.32</td>
<td>7.88±0.15</td>
<td>8.24±0.23</td>
<td>8.10±0.17</td>
</tr>
</tbody>
</table>

SCr=serum creatinine; SCa²⁺=serum calcium cation.
Data are means ± SE; n=5 in each group in day 30. *P<0.05 vs control.
Fig. 1.

(a) Urinary Albumin to creatinine ratio (μg/mgCr) over different treatment groups and time points (Day0, Day3, Day5, Day15, Day30). The graph shows significant differences between the control and treated groups, with Oxacalciotrol and Telmisartan showing moderate effects.

(b) Systolic blood pressure in mmHg over the same time points. The graph indicates a trend towards increased blood pressure in the treated groups compared to the control.

Fig. 1.
Fig. 1. Control Vehicle
Oxacalcitriol Telmisartan Combination

**c**

**d**

![Sclerotic glomeruli/total glomeruli (%)](chart1.png)

**e**

![GFR ratio (%)](chart2.png)

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**Fig. 1.**
Fig. 2.

(a) Immunofluorescence images of Nephrin and Podocin on Day 5, Day 15, and Day 30 for Control, Vehicle, Oxacalcitriol, Telmisartan, and Combination groups.

(b) Bar graphs showing Nephrin and Podocin scores on Day 5 and Day 15 for each group.

* indicates significant difference compared to Control group.
# indicates significant difference compared to the Vehicle group.
*** indicates highly significant difference compared to the Control group.

Nephrin and Podocin expressions were evaluated by immunofluorescence staining.
Fig. 2.
**Fig. 3.**

**a**

Image showing a renal tissue section with免疫 staining for WT1 and Synaptopodin.

**b**

Bar graph showing TUNEL positive cells/glomerulus (%) for different treatments: Control, Vehicle, Oxacalcitriol, Telmisartan, Combination. The graph indicates a significant increase in TUNEL positive cells in the Oxacalcitriol group compared to other groups.

**c**

Images of kidney sections stained for WT1, Synaptopodin, and merged images for different treatments: Control, Vehicle, Oxacalcitriol, Telmisartan, Combination. The images show the distribution and intensity of staining for each treatment.

**d**

Bar graph showing the number of WT1-positive cells/glomerular volume for different treatments: Control, Vehicle, Oxacalcitriol, Telmisartan, Combination. The graph shows a significant increase in WT1-positive cells in the Oxacalcitriol group compared to other groups.

* indicates a significant difference compared to the control group. # indicates a significant difference compared to the Vehicle group.
Fig. 4.