Title page

Title: The synergistic effect of mizoribine and a direct renin inhibitor, aliskiren, on unilateral ureteral obstruction-induced renal fibrosis in rats

Running title: Aliskiren and mizoribine attenuates renal fibrosis

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ABSTRACT

Purpose: Renal fibrosis is the major histopathological change observed in a variety of renal disorders and closely related to renal dysfunction. Unilateral ureteral obstruction (UUO) is a well-established model of experimental renal disease, which results in tubulointerstitial fibrosis. Previous studies have shown that both aliskiren and mizoribine (MZR) ameliorate UUO-induced renal fibrosis. However, the protective effect of combination therapy with aliskiren and MZR against renal fibrosis is unknown. In this study, we investigated the synergistic effects of combination therapy with aliskiren and MZR on UUO-induced fibrosis in rats.

Materials and Methods: Sprague-Dawley male rats underwent UUO, followed by treatment with either aliskiren, MZR, or both drugs. Kidney samples were fixed for histopathology and immunohistochemistry of myofibroblasts (α-smooth muscle actin; α-SMA) and macrophages (ED-1). Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to measure the expression of α-SMA, transforming growth factor-β1 (TGF-β1), osteopontin (OPN), monocyte chemotactic protein-1 (MCP-1), and renin.

Results: The scores for tubular dilatation, interstitial volume, and α-SMA expression following UUO were significantly reduced by combination therapy compared with monotherapy with either aliskiren or MZR. Combination therapy also caused a significant decrease in the number of ED-1 positive cells and expression of TGF-β1 gene compared with monotherapy with either drug (both \( p < 0.05 \)). Combination therapy also decreased the expression of OPN and MCP-1 gene (\( p < 0.05 \)).

Conclusion: Combination therapy with aliskiren and MZR provides increased renal protection
against renal fibrosis and UUO-induced inflammation.
INTRODUCTION

Renal tubulointerstitial fibrosis is the major histopathological change observed in a variety of renal disorders and closely related to renal dysfunction\(^1\). Several studies have demonstrated that infiltration of macrophages and activation of the renin-angiotensin system (RAS) plays important roles in the pathogenesis of renal fibrosis\(^2\). Unilateral ureteral obstruction (UOO) is a well-established model of experimental renal disease, which results in tubulointerstitial fibrosis\(^3, 4\).

Many cellular and molecular events occur in UUO kidneys during initiation and progression of renal injury, including inflammation and fibrosis\(^5\). Macrophages and fibroblasts are rarely observed in healthy kidneys. In contrast, a large number of blood-derived macrophages and activated alpha-smooth muscle actin (α-SMA)-positive fibroblasts accumulate in the tubulointerstitial space in UUO kidneys. This leads to renal inflammation and fibrosis. The associated increase in angiotensin II (Ang II) and activation of the RAS in various kidney diseases also play crucial roles by up-regulating fibrogenic factors, such as transforming growth factor-beta 1 (TGF-β\(^1\))\(^5\). Ang II also causes constriction of the efferent arterioles in the kidney, thereby increasing intraglomerular pressure which results in kidney injury\(^6\). RAS inhibition with an angiotensin-converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) could ameliorate renal tubulointerstitial fibrosis caused by UUO\(^7\). A novel oral direct renin inhibitor (DRI), aliskiren, was recently reported to attenuate renal fibrosis in UUO rats by inhibiting the expression of Ang-stimulated TGF-β\(^1\) and decreasing the plasma renin activity\(^8, 9\). In March 2007, aliskiren was the first oral DRI approved by the US Food and Drug Administration for treatment of hypertension with less side effects such as
angioedema and hyperkalemia compared with ACE inhibitor, either as monotherapy, or in combination with other antihypertensive drugs\textsuperscript{10}. A previous study showed that aliskiren and perindopril were equally effective in reducing albumin and glomerulosclerosis in diabetic animals, although aliskiren reduced interstitial fibrosis to a greater extent than perindopril\textsuperscript{11}.

Because macrophages play an important role in the fibrosis evolution, strategies to arrest the proliferation of these cells have been proposed. Recent studies in UUO rats have demonstrated that mizoribine (MZR), a purine nucleotide analog isolated from \textit{Eupenicillium brefeldianum}\textsuperscript{12}, suppresses macrophage infiltration and ameliorates the tubulointerstitial fibrosis associated with obstructive nephropathy\textsuperscript{13,14}. These results indicate that MZR has the potential to prevent interstitial fibrosis.

These earlier studies therefore showed that aliskiren and MZR ameliorate renal tubulointerstitial fibrosis in UUO kidneys through different mechanisms. Based on these experimental evidences, we aimed to investigate the possible synergistic effect of combination therapy with aliskiren and MZR on UUO-induced renal fibrosis.
MATERIALS AND METHODS

Animals. Studies were performed on male Sprague-Dawley rats weighing between 200–250 g. The rats were housed in cages in a temperature- and light-controlled environment at the Juntendo University Animal Care facility. The protocol used in this study was approved by the Juntendo University Animal Care Committee.

Experimental design. UUO was performed as previously described9, 13). In brief, the left kidney and ureter were exposed via a flank incision under light anesthesia with isoflurane. The left ureter was ligated with 7-0 silk sutures at two points. The incision was then closed, and rats were fed a standard basal diet and given tap water. Sham animals underwent identical surgical procedures in which the left ureter was simply manipulated.

The rats were divided into the following five groups: 1) sham-vehicle, in which the rats underwent sham operations and were treated with vehicle (n = 5); 2) UUO-vehicle, in which the rats underwent UUO and were treated with vehicle (n = 5); 3) UUO-MZR, in which the rats underwent UUO and were treated with MZR (n = 5); 4) UUO-aliskiren, in which the rats underwent UUO and were treated with aliskiren (n = 5); and 5) UUO-combination therapy, in which the rats underwent UUO and were treated with aliskiren and MZR (n = 5).

Aliskiren powder was used in the study. To deliver solutions continuously over 14 days a Model 2ML2 Alzet® mini-osmotic pump was subcutaneously implanted in each rat following 1 day of UUO. The pumps were filled with normal saline containing aliskiren, which was administered at a dose of 20 mg/kg daily. MZR at a dose of 10 mg/kg daily was intraperitoneally administered once a
day for 14 days from the day following UUO. The doses of these two drugs were determined by previous study. To evaluate plasma renin activity, whole-blood samples were collected from the inferior artery after sacrifice of the rat under anesthesia. All the rats were sacrificed the day after the final administration of the drugs.

**Histopathological and immunohistochemical analysis.** Both kidneys were removed, fixed in 10% formalin in PBS, and then embedded in paraffin. Sections (4 μm) were stained with hematoxylin-eosin (HE) to assess the grade of tubulointerstitial damage. A standard point counting method, modified from previous reports, was used to determine the histological changes following UUO. In brief, 10 nonoverlapping fields from each section of the renal cortex were photographed at high magnification (×400). A grid containing 100 (10×10) sampling points was superimposed on each photograph. Points falling on glomerular structures or large vessels were excluded from the total count. The tubular dilatation score was expressed as the percentage of points that overlaid dilated tubular spaces. Interstitial fibrosis volume was evaluated by blue staining with Elastica-Masson trichrome. Ten nonoverlapping fields from each section of the renal cortex were photographed. The interstitial area superimposed on the photograph was measured using the KS400 Image Analysis System (Carl Zeiss, Oberkohen, Germany), and the percentage of interstitium per unit area was calculated.

The immunohistochemical studies were performed on the paraffin-embedded sections as previously described. All the blocks were stained using a Ventana Ultraview DAB detection kit in a Ventana BenchMark XT processor (Ventana, Tucson, AZ, USA). As a negative control, the primary
antibody was replaced with normal rabbit IgG, without staining. To evaluate infiltration of interstitial monocytes and macrophages, mouse antirat ED-1 antibody (monocyte/macrophage marker, clone ED-1; AbD Serotec, Kidlington, UK) was used for the primary reaction, followed by a secondary reaction with biotinylated goat antimouse IgG (Vector Laboratories, Burlingame, CA, USA). The 3,3'-diaminobenzidine reaction was then performed on the section using a commercially available kit (Dako, Carpinteria, CA, USA) with hematoxylin as the counterstain. The number of ED-1 positive cells was determined in 10 randomly chosen ×400 fields within the same section of kidney from an individual animal. Monoclonal mouse antihuman α-SMA (Dako; final dilution 1:100) was used to evaluate myofibroblast differentiation. All the samples were evaluated using the KS400 Image Analysis System (Carl Zeiss, Oberkochen, Germany). The number of areas stained for α-SMA was expressed as a percentage of the total area in 10 fields of each section under ×400 magnification.

**RT-PCR.** Real-time quantitative Reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the expression of α-SMA, TGF-β1, osteopontin (OPN), monocyte chemotactic protein-1 (MCP-1), and renin in the renal cortex using the Taq Man system according to the manufacturer’s protocols (Applied Biosystems, Foster City, CA, USA). TaqMan probe-based quantitative RT-PCR was performed using cDNA synthesized from kidney biopsy RNA prepared using the High Capacity cDNA Reverse Transcription Kit and analyzed using the default protocols of the 7500 Fast Real-Time PCR system. The expression of each gene was normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using the standard curve method. Primers and probes for α-SMA (Rn01759928_m1), TGF-β1 (Rn00572010_m1), OPN
(Rn01449972_m1), MCP-1 (Rn00580555_m1), and renin (Rn00561847_m1) were prepared using TaqMan® Gene Expression Assays.

**Statistical analysis.** Continuous variables were expressed as mean ± SD. One-way ANOVA was used to examine differences between the groups, followed by post-hoc multiple comparisons using the Bonferroni method. All the statistical analyses were performed using SPSS v.18.0 (IBM Corp., Armonk, NY, USA). Statistical significance was defined as $p < 0.05$. 
RESULTS

Combination therapy with aliskiren and MZR attenuated histological changes in UUO kidneys.

Ureter ligation generated a model of renal interstitial fibrosis, characterized by tubular atrophy and interstitial matrix deposition in the obstructed kidneys. In contrast, no histological changes were observed in the sham-operated kidneys. Figure 1 shows representative histological finding with either hematoxylin-eosin (HE), Elastica-Masson trichrome, α-SMA, or ED-1 staining in kidney tissue of UUO rats treated with vehicle, MZR, aliskiren, or combination therapy. The UUO kidney treated with vehicle showed severe tubular dilatation, tubular atrophy, and widened interstitial spaces containing a greater number of interstitial cells and infiltrating leukocytes. These changes were observed in the entire cortex, although the severity of the changes was not homogeneously distributed. Aliskiren administration caused a significant attenuation in tubulointerstitial damage following UUO (Fig. 1C), with the score for tubular dilatation being significantly reduced by 50.3% (from 30.6% to 15.2%, \( p < 0.05 \), Fig. 2A). A similar reduction in tubular dilatation score (47.3%) was observed with MZR (Fig. 1D). However, as shown in Fig. 1E, combination therapy caused a significantly greater reduction in tubular dilatation score (78.7%, \( p < 0.05 \)).

The interstitial volume of UUO kidneys was significantly increased compared with that of sham-operated kidneys. Aliskiren administration blunted the increase in interstitial volume score by 41.7% (from 28.3 to 16.5%, \( p < 0.05 \), Fig. 2B); MZR elicited a similar effect (41.1%). Combination therapy showed a significantly greater effect (78.1%, \( p < 0.05 \)) than monotherapy with either drug.
Combination therapy with aliskiren and MZR decreased expression of α-SMA in UUO kidneys.

We next examined the effect of combination therapy with aliskiren and MZR on interstitial myofibroblasts measured as expression of α-SMA. α-SMA was mainly expressed in vascular smooth muscle cells in rats. This contrasted with a high number of cells expressing α-SMA surrounding the peritubular and periglomerular spaces following UUO (Fig. 1L). Aliskiren significantly reduced the α-SMA expression score in the cortical interstitium of UUO rats by 18.0% (from 20.6 to 16.9, \( p < 0.05 \), Fig. 2C). MZR was more effective than aliskiren (24.3%; Fig. 1M). In contrast, combination therapy caused a significantly greater reduction in score (70.3%, \( p < 0.05 \)) than monotherapy with either drug (Fig. 1O).

RT-PCR analyses of the kidney tissue extracts also revealed that α-SMA expression in UUO kidneys was significantly increased. Combination therapy with aliskiren and MZR elicited considerable attenuation of this increased expression (Fig. 3A). It was more effective than monotherapy with either drug. However, this difference between monotherapy and combination therapy was not statistically significant.

Combination therapy with aliskiren and MZR reduced infiltration of monocytes and macrophages in UUO kidneys

Infiltration of ED-1-positive monocytes or macrophages in UUO kidneys was observed in the current study, similar to that reported previously \(^{11}\). Pretreatment with aliskiren resulted in a mild reduction in the number of infiltrating ED-1-positive cells by 36.3% (from 56.5 to 36.0, \( p < 0.05 \), Fig.
2D). As shown in Fig. 2D, MZR was more effective than aliskiren (43.2%). Combination therapy showed a greater inhibitory effect (81.9%) than monotherapy with either drug ($p < 0.05$, Fig. 1T).

RT-PCR analyses of the kidney tissue extracts showed that OPN expression in obstructed kidneys was significantly increased. Combination therapy with aliskiren and MZR significantly attenuated the increase in OPN expression (Fig. 3B); it had a significantly greater effect than monotherapy with either drug. Similarly, combination therapy significantly decreased MCP-1 expression. Combination therapy was more effective than monotherapy with either drug (Fig. 3C).

**Effect of each treatment on expression of TGF-β1 mRNA in UUO kidneys**

Figure 4 shows that the expression of TGF-β1 mRNA was increased in UUO kidneys. Combination therapy with aliskiren and MZR had a significant ameliorating effect on UUO-induced expression of TGF-β1 mRNA. Combination therapy was more effective than monotherapy with either drug.

**Plasma renin activity and renal renin mRNA expression in UUO kidneys**

Plasma renin activity was higher in UUO rats than in sham-operated rats. Aliskiren administration in UUO rats was associated with a significant decrease in plasma renin activity compared with vehicle-treated rats (Fig. 5A). UUO rats treated with combination therapy also had a significantly lower plasma renin activity level than vehicle-treated rats.

Renal expression of renin mRNA was higher in UUO rats than in sham-operated rats. Both aliskiren and combination therapy also increased renal expression of renin mRNA (Fig. 5B).
DISCUSSION

To our knowledge, this study is the first to demonstrate that combination therapy with aliskiren and MZR has a synergistic effect for attenuating UUO-induced renal fibrosis. We think that the treatment one day after the induction of UUO is a true treatment rather than prevention. The rationale for this combined therapy is based on different mechanisms of actions of these two drugs. The mechanisms responsible for renal fibrosis involve several contributory steps. Mechanical stretching of tubules dilated by downstream obstruction to urine flow causes injury to epithelial cells, which activates the local RAS, which in turn, stimulates the expression of TGF-β1. These changes lead to the recruitment of macrophages to the interstitium and generation of chemoattractants and adhesion molecules. Stimulus by cytokines, such as TGF-β1 produced by macrophages and other cells, causes fibroblasts to synthesize stress fibers and undergo further differentiation to become myofibroblasts, leading to progressive interstitial fibrosis\textsuperscript{15, 16}.

Recent studies showed that aliskiren ameliorated renal inflammation and fibrosis in animal models of UUO\textsuperscript{8-9}. Inhibition of RAS in various models of kidney injury also decreased the inflammatory process, including infiltration of macrophages and elevation of chemoattractants, such as OPN and MCP-1\textsuperscript{17}. Macrophage infiltration and chemoattractants have important roles in the inflammatory process in cases of UUO\textsuperscript{18}, and aliskiren decreases inflammation, macrophage infiltration, OPN expression, and MCP-1 expression in UUO kidneys\textsuperscript{8}. In addition, activation of RAS, particularly Ang II, increases the expression of TGF-β1 and α-SMA in kidneys\textsuperscript{5}. In progressive renal fibrosis, TGF-β1 plays an important role in transdifferentiation of tubular epithelial cells to α-SMA-positive
myofibroblasts\(^{19}\)). In particular, intrarenal Ang II may act in an autocrine and/or paracrine manner and lead to suppression of TGF-\(\beta\)1 expression in the kidney\(^{20}\)). Aliskiren suppresses intrarenal Ang II, thereby resulting in suppression of TGF-\(\beta\)1 expression in UUO kidneys and decreased tubulointerstitial fibrosis and \(\alpha\)-SMA immunoreactivity\(^9\)).

On the other hand, MZR is a selective inhibitor of inosine monophosphate dehydrogenase, which is the key enzyme in \textit{de novo} synthesis of purine nucleotides\(^{21}\)). MZR is an immunosuppressant with effects similar to that of azathioprine and mycophenolate mofetil but with the advantage of having very few side effects, such as diarrhea and appetite loss, even with long-term administration. MZR was developed in Japan, and it has been mainly used in Asian countries. In addition to its use following renal transplantation, recent studies have demonstrated the efficacy and safety of MZR in the treatment of renal diseases, including lupus nephritis, IgA nephropathy, and childhood nephrotic syndrome\(^{22–24}\)). MZR has also been reported to decrease macrophage infiltration, OPN secretion, and TGF-\(\beta\)1 expression in an animal model of UUO, with these decreases associated with improved histopathology\(^{13}\)). It has been suggested that macrophage accumulation within the tubulointerstitium may represent a more important pathway of progressive renal injury\(^{25}\)). Macrophages can synthesize extracellular matrix proteins, such as collagen and fibronectin, which may cause interstitial fibrosis\(^{26}\)). In addition, infiltrating macrophages secrete soluble factors, such as TGF-\(\beta\)1\(^{27}\)). Although the direct action of MZR on macrophages has not yet been described in the literature, an alternative explanation may be that MZR acts by regulating the release of protein chemoattractants. This possibility is supported by the finding in the present study that treatment with MZR in UUO rats
caused a significant reduction in the expression of OPN in renal cortical tubules. Giachelli speculated that OPN is an important proinflammatory chemoattractant in tubulointerstitial disease\textsuperscript{28}, and there is evidence that expression of OPN precedes histological evidence of tubule injury and correlates with subsequent accumulation of monocytes and macrophages\textsuperscript{29}. It is therefore possible that MZR modulates macrophage migration by reducing the increase in OPN expression in tubular epithelial cells.

In this study, the scores for tubular dilatation, interstitial volume, $\alpha$-SMA expression, and ED-1 positive cells decreased following treatment with either aliskiren, MZR, or combination therapy. RT-PCR confirmed that treatment with aliskiren and MZR inhibited the expression of $\alpha$-SMA, TGF-\textbeta{}1, OPN, and MCP-1. In particular, monotherapy with both aliskiren and MZR had similar inhibitory effects on renal fibrosis, whereas combined therapy with these two drugs showed a significantly greater effect on UUO-induced renal fibrosis than monotherapy with either drug. Similar to previous reports, our study also found that combination therapy with aliskiren and MZR or monotherapy with aliskiren increased expression of renin mRNA in UUO kidneys, though a previous study demonstrated that renin activity may be decreased by the intracellular action of aliskiren\textsuperscript{8}. Taken together these results show that both aliskiren and MZR have a significant, but not complete, renoprotective effect on renal fibrosis. Theoretically, aliskiren can decrease both Ang II formation and plasma renin activity despite the fact that it also causes incomplete inhibition of renin activity by interacting with the active site of the enzyme\textsuperscript{30}. On the other hand, MZR may have beneficial effects on several other processes, particularly reducing macrophage infiltration, which
contributes to interstitial fibrosis. However, MZR has only a minimal inhibitory effect on expression of angiotensin-stimulated TGF-β, which plays an important role in renal fibrosis. Therefore, appropriate combination therapy with aliskiren and MZR may have a synergistic effect on renal fibrosis. Theoretically, this suggests that the two drugs act mechanistically through different pathways.

In summary, interstitial renal fibrosis is a common final pathway leading to end-stage renal failure, irrespective of the nature of the initial renal injury. Our results provide compelling evidence that combination therapy with aliskiren and MZR results in increased renal protection against renal fibrosis and UUO-induced inflammation. In view of this synergistic protective effect, we suggest that combination therapy with aliskiren and MZR can be a clinical choice of early intervention for progressive renal dysfunction.

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DISCLOSURE

None.
REFERENCES


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Figure legends

Figure 1: **Combination therapy with aliskiren and MZR attenuated tubulointerstitial changes in obstructed kidneys by different types of staining.**

Representative micrographs showing hematoxylin-eosin (HE; A–E), Elastica-Masson trichrome (Elastica-Masson trichrome; F–J), α-smooth muscle actin (α-SMA; K–O), and ED-1 (P–T) staining in kidney tissue from normal rats that underwent a sham operation (A,F,K,P) or rats with Unilateral ureteral obstruction (UUO) and were then treated with either vehicle (B,G,L,Q), MZR (C,H,M,R), aliskiren (D,I,N,S), or combination therapy with aliskiren and MZR (E,J,O,T). Original magnification ×200.

Figure 2: **Combination therapy with aliskiren and MZR attenuated the scores of tubulointerstitial damage in obstructed kidneys.**

The scores for tubular dilatation (A), interstitial volume (B), α-SMA expression (C), and ED-1 positive cells in obstructed kidneys (D). * indicates $p < 0.05$ vs. vehicle-treated UUO rats. ** indicates $p < 0.01$ vs. vehicle-treated UUO rats. # indicates $p < 0.05$ vs. aliskiren- or MZR-treated UUO rats. n = 5 for each group. The bar represents mean ± SD. Abbreviations: U, UUO treated with vehicle; U + M, UUO treated with mizoribine; U + A, UUO treated with aliskiren; U + M + A, UUO treated with a combination of aliskiren and MZR.

Figure 3: **RT-PCR analysis showed that combination therapy with aliskiren and MZR was**
more effective than monotherapy with either aliskiren or MZR for blocking increased expression of α-SMA, OPN, and MCP-1 in obstructed kidneys.

Expression of α-SMA, OPN, and MCP-1 in sham-operated kidneys or UUO kidneys treated with either vehicle, aliskiren, MZR, or combination therapy. Representative mRNA analyses (A, B, C) show the expression in obstructed kidneys after various treatments. * indicates $p < 0.05$ vs. vehicle-treated UUO rats. ** indicates $p < 0.01$ vs. vehicle-treated UUO rats. # indicates $p < 0.05$ vs. aliskiren- or MZR-treated UUO rats. n = 5 for each group. The bar represents mean ± SD. The abbreviations for the treatments are the same as those shown in Fig. 2.

Figure 4: Combination therapy with aliskiren and MZR has a synergistic effect that reduces the expression of TGF-β1 in obstructed kidneys.

Expression of TGF-β1 in sham-operated kidneys or UUO kidneys treated with either vehicle, aliskiren, MZR, or combination therapy. Representative mRNA analyses show the expression in obstructed kidneys after various treatments. * indicates $p < 0.05$ vs. vehicle-treated UUO rats. ** indicates $p < 0.01$ vs. vehicle-treated UUO rats. # indicates $p < 0.05$ vs. aliskiren- or MZR-treated UUO rats. n = 5 for each group. The bar represents mean ± SD. The abbreviations for the treatments are the same as those shown in Fig. 2.

Figure 5:

Plasma renin activity (A) and expression of renin mRNA (B) in obstructed kidneys of UUO rats
treated with either vehicle, aliskiren, MZR, or combination therapy. * indicates $p < 0.05$ vs.
vehicle-treated rats with UUO. $n = 5$ for each group. The bar represents mean ± SD. The
abbreviations for the treatments are the same as those shown in Fig. 2.