# The Synergistic Effect of Mizoribine and a Direct Renin Inhibitor, Aliskiren, on Unilateral Ureteral Obstruction Induced Renal Fibrosis in Rats

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**Purpose:** Renal fibrosis, the major histopathological change in various renal disorders, is closely related to renal dysfunction. Unilateral ureteral obstruction is a well established model of experimental renal disease that results in tubulointerstitial fibrosis. Previous studies showed that aliskiren and mizoribine ameliorated unilateral ureteral obstruction induced renal fibrosis. However, to our knowledge the protective effect of combination therapy with aliskiren and mizoribine against renal fibrosis is unknown. We investigated the synergistic effects of aliskiren and mizoribine combination therapy on unilateral ureteral obstruction induced fibrosis in rats.

**Materials and Methods:** Male Sprague Dawley® rats underwent unilateral ureteral obstruction followed by aliskiren and/or mizoribine treatment. Kidney samples were fixed for histopathology and immunohistochemistry of myofibroblasts (α-SMA) and macrophages (ED-1). Real-time quantitative reverse transcription-polymerase chain reaction was performed to measure α-SMA, TGF-β1, osteopontin, MCP-1 and renin expression.

**Results:** After unilateral ureteral obstruction the tubular dilatation, interstitial volume and α-SMA expression scores were significantly decreased by combination therapy compared with monotherapy with aliskiren or mizoribine. Combination therapy caused a significant decrease in the number of ED-1 positive cells and in TGF-β1 gene expression compared with monotherapy with either drug (each $p < 0.05$). Combination therapy also decreased OPN and MCP-1 gene expression ($p < 0.05$).

**Conclusions:** Aliskiren and mizoribine combination therapy provides increased renal protection against renal fibrosis and unilateral ureteral obstruction induced inflammation.

**Key Words:** kidney, ureteral obstruction, aliskiren, bredinin, drug synergism

**Abbreviations and Acronyms**

α-SMA = α-smooth muscle actin
Ang = angiotensin
MCP-1 = monocyte chemotactic protein-1
MZR = mizoribine
OPN = osteopontin
RAS = renin-angiotensin system
RT-PCR = reverse transcription-polymerase chain reaction
TGF-β1 = transforming growth factor-β1
UUO = unilateral ureteral obstruction

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Renal tubulointerstitial fibrosis, the major histopathological change in various renal disorders, is closely related to renal dysfunction.[1] Several studies demonstrated that macrophage infiltration and RAS activation have important roles in the pathogenesis of renal fibrosis.[2] UUO is a well established model of experimental renal disease that results in tubulointerstitial fibrosis.[3,4]

Many cellular and molecular events occur in UUO kidneys during the initiation and progression of renal

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injury, including inflammation and fibrosis.[4] Macrophages and fibroblasts are rarely observed in healthy kidneys. In contrast, a large number of blood derived macrophages and activated α-SMA positive fibroblasts accumulate in the tubulointerstitial space in UUO kidneys, leading to renal inflammation and fibrosis. The associated Ang II increase and RAS activation in various kidney diseases have crucial roles by up-regulating fibrogenic factors such as TGF-β1.[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 Ang-converting enzyme inhibitor or Ang receptor blocker could ameliorate the renal tubulointerstitial fibrosis caused by UUO.[7]

The novel oral direct renin inhibitor aliskiren was recently reported to attenuate renal fibrosis in UUO rats by inhibiting the expression of Ang stimulated TGF-β1 and decreasing plasma renin activity.[8,9] In March 2007 aliskiren was the first oral direct renin inhibitor approved by the United States Food and Drug Administration for hypertension with fewer side effects (such as angioedema and hyperkalemia) than Ang-converting enzyme inhibitor when used as monotherapy or in combination with other antihypertensive drugs.[10] Aliskiren and perindopril were equally effective in decreasing albumin and glomerulosclerosis in diabetic animals, although aliskiren reduced interstitial fibrosis to a greater extent than perindopril.[11]

Because macrophages have an important role in the fibrosis evolution, strategies were proposed to arrest the proliferation of these cells. Recent studies in UUO rats demonstrated that MZR, a purine nucleotide analogue isolated from Eupenicillium brefeldianum,[12] suppresses macrophage infiltration and ameliorates the tubulointerstitial fibrosis associated with obstructive nephropathy.[13,14] These results indicate that MZR has the potential to prevent interstitial fibrosis.

These earlier studies showed that aliskiren and MZR ameliorate renal tubulointerstitial fibrosis in UUO kidneys through different mechanisms. Based on this experimental evidence we investigated the possible synergistic effect of combination therapy with aliskiren and MZR on UUO induced renal fibrosis.

## MATERIALS AND METHODS

### Animals

Studies were performed in male Sprague Dawley rats weighing between 200 and 250 gm. The rats were housed in cages in a temperature and light controlled environment at 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 described.[9,13] Briefly, the left kidney and ureter were exposed via a flank incision using light anesthesia with isoflurane. The left ureter was ligated with 7-zero silk sutures at 2 points and the incision was closed. The rats received a standard basal diet and tap water. Sham operated rats underwent identical surgical procedures in which the left ureter was simply manipulated.

The rats were divided into 5 groups of 5 each, including group 1—sham operation and vehicle treatment, group 2—UUO and vehicle treatment, group 3—UUO and MZR treatment, group 4—UUO and aliskiren treatment, and group 5—UUO and aliskiren plus MZR treatment.

Aliskiren powder was used. To deliver solutions continuously during 14 days we subcutaneously implanted a Model 2ML2 mini-osmotic pump (Alzet®) in each rat after 1 day of UUO. The pumps were filled with normal saline containing aliskiren, administered at a dose of 20 mg/kg daily. MZR at a dose of 10 mg/kg daily was intraperitoneally administered once daily for 14 days starting the day after UUO. The doses of these 2 drugs were previously determined.[8,9,13,14] To evaluate plasma renin activity whole blood samples were collected from the inferior artery after sacrifice of the rat under anesthesia. All rats were sacrificed the day after final drug administration.

### Histopathological and Immunohistochemical Analysis

The kidneys were removed, fixed in 10% formalin in phosphate buffered saline and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin to assess the grade of tubulointerstitial damage. To determine histological changes after UUO we used a standard point counting method modified from a previous study.[9] Briefly, 10 nonoverlapping fields from each section of the renal cortex were photographed at 400× magnification. 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 is expressed as the percent of points that overlaid dilated tubular spaces. Interstitial fibrosis volume was evaluated by blue staining with Elastica-Masson trichrome. Ten nonoverlapping fields per renal cortex section were photographed. The interstitial area superimposed on the photograph was measured using the KS400 Image Analysis System (Carl Zeiss, Oberkochen, Germany) and the percent of interstitium per unit area was calculated.

Immunohistochemical studies were performed on paraffin embedded sections as previously described.[8,9] All blocks were stained using an ultraView™ DAB Detection Kit in a BenchMarkXT processor (Ventana™). As a negative control, the primary antibody was replaced with normal rabbit IgG without staining. To evaluate interstitial monocyte and macrophage infiltration mouse antirat ED-1 antibody (monocyte/macrophage marker, clone ED-1, AbD Serotec, Kidlington, United Kingdom)