Annexures
DSC for Aliskiren

Annexures
Temperature programme
Start temperature 40 °C
Temperature Rate [°C/min]=10 °C
Hold temperature [°C]= 240
Hold time [min]=

File name : 2010-02-05OLMESARTAN
MEDOXOMIL
Detector : DSC60
Acquisition date-2010-02-05
Sample name – OLMESARTAN
MEDOXOMIL
Sample weight – 3.00 mg

DSC for Olmesartan
DSC for Glimepiride
# CERTIFICATE OF ANALYSIS

**Product**
- GARCINOL

**Batch No.**
- F70223

**A. R. No.**
- BLP08C0047

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**REMARKS: COMPLIES WITH OUR SPECIFICATIONS**

For Sami Labs Limited

Prepared By: [Signature]
- Irfan Pasha
  Senior Scientist – Analytical R&D

Checked By: [Signature]
- And Kumar Thakan
  Senior Manager – Analytical Research

Approved By: [Signature]
- Dr. Beena Bhat
  Head – Phytochemistry

January 5, 2008

Corporate Office: 19/1 & 19/2, I Main, II Phase, Peenya Industrial Estate, Bangalore - 560 058.
### QUALITY CONTROL DEPARTMENT
SANAT PRODUCTS LIMITED

### CERTIFICATE OF ANALYSIS

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*Legend - NLT-Not less than, NMT-Not more than, Cfu - Colony forming unit / gram*

**ANALYSED BY**
[Signature]

Regd. Office & works: B-8, Industrial Area, Sikandarpur, (U.P. 203205 (INDIA) Tel. (05735)/223465/222137
FAX: 05735-222136 Marketing & Administrative office: 11th floor, Sagar Plaza, Delhi - 110092, INDIA.'
**RESULTS OF ANALYSIS**

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- Reddish brown round plain uncoated tablets.

**Remarks:** Party asked for the above tests only

Date: Tuesday, August 10, 2010

Person In Charge

176
Publications
PATENT APPLICATION

We have sent invention related to this thesis for patent filing. The contents of this thesis should be kept confidential and should not be disclosed to anyone other than reviewers till it is published

Prof. (Dr.) B.P. Srinivasan           Ms. Sonia Gandhi
(Inventor)                            (Inventor)
Effective blockade of RAAS by combination of aliskiren and olmesartan improves glucose homeostasis, glomerular filtration rate along with renal variables in streptozotocin induced diabetic rats

Sonia Gandhi *, B.P. Srinivasan, Atul Sureshrao Akarte

Delhi Institute of Pharmaceutical Sciences and Research, Pushp Vihar, Sector-3, MB Road, New Delhi 110 017, India

Abstract

The present study aims to investigate the combined and individual treatment of aliskiren and olmesartan for 8 weeks in streptozotocin induced diabetic rats provide an effective blockade of RAAS by improving glucose homeostasis, glomerular filtration rate along with renal variables thereby delaying the progression of the disease. Streptozotocin induced diabetic rats were administered with aliskiren (10 mg/kg/day), olmesartan (6 mg/kg/day) alone and in combination. To identify the glucose homeostasis, translocation of glucose transporter proteins in liver and muscle was observed by their expression after treatment. Glomerular filtration rate is estimated using serum creatinine, cystatin C and beta 2 microglobulin. This study also examined the effects of combination and monotherapy on various renal variables viz. albumin, total proteins, TGF-β, TNF-α, VEGF, nitric oxide, adiponectin and erythropoietin. In addition, histopathological and anti-apoptotic profile of kidney was also investigated. The present study indicates that dual blockade of RAAS improved glucose homeostasis and confirms the nephroprotective effects of the combined treatment of aliskiren and olmesartan independent of their antihypertensive property in the STZ induced diabetes. In addition, its antifibrotic, antiproteinuric effects indicate that combination treatment might be potential as an important therapeutic option for chronic fibrotic diseases in renal complications.

Keywords: Antifibrotic Antiproteinuric Glucose homeostasis RAAS Glomerular filtration rate

1. Introduction

Dysregulation of the renin angiotensin aldosterone system (RAAS) has an important role in the development of target organ damage in diabetes mellitus (Remuzzi et al., 2006; Ingelfinger, 2008; Price et al., 1999). Therefore, drugs targeting RAAS suppression have been most widely used for preventing or delaying the progression of target organ damage (Remuzzi et al., 2006). Although most of the RAAS blockers are not completely effective in preventing or reversing diabetic complications, which could be due to the stimulation of renin that results from the negative feedback loop associated with decreased angiotensin II activation. Thus, direct renin inhibition provides a more logical approach to create a complete blockade of RAAS activity without a rebound increase in plasma renin activity (PRA). The blockade of RAAS through the inhibition of renin was conceptualized years ago (Skeggs et al., 1957). Alikiren is the first drug of a new class of agents known as renin inhibitors, which directly inhibit renin in order to decrease PRA (Cohen, 2007). Unlike previous renin inhibitors, aliskiren has better oral bioavailability and an extended half-life (Villamil et al., 2007).

The most recent reports for aliskiren treatment revealed effective organ protection related to an anti-hypertensive effect (Stanton et al., 2003; Oparil et al., 2007; Pilz et al., 2005; Kelly et al., 2007). The aliskiren treatment through monotherapy or combination therapy with other RAAS blockades has been shown to have protective renal and cardiac benefits in human and animal studies. Renin, in addition to its classic role of converting angiotensinogen into angiotensin I (Ang I), could exert a direct effect through its own receptor (Nguyen et al., 1996, 2002, 2003). The binding of prorenin/renin to prorenin receptor (P) RR produces angiotensin-independent intra-cellular effects, including phosphorylation and activation of the extracellular signal regulated kinases 1 and 2 (ERK1/2), and increase in plasminogen-activator inhibitor-1 (PAI-1) release. Inhibition of (P) RR attenuated mesangial cell proliferation and reduced associated fibrotic factor release (He et al., 2009). This non-enzymatic pathway of prorenin/renin plays a role in renal pathological conditions (Birkenhager and Staessen, 2007). However, aliskiren does not inhibit prorenin activation. Regardless of the precise explanation, aliskiren does not eliminate the need for multi-drug regimens for resistant hypertension and renal complications in diabetic patients. Hence, ARBs serve as a very good substitute for the susceptible patients especially on ACEI thereby producing effective results and lower side effects especially troubling cough as a result of the increase in kinins (Nyathani et al., 2011). Olmesartan...
medoxomil, selective AT1 receptor blocker has a good oral bioavailability, is not metabolized by the cytochrome P450 enzyme pathway, is competitively priced and well tolerated. Hence, olmesartan is the drug of choice if the patient has compromised liver function (Craig and Stitzel, 2003).

Dual RAAS blockade at different sites in the RAAS pathway theoretically provide additive protective effects by further reducing systemic and local levels of some or all angiotensin peptides, which could further inhibit formation of the Ang II: AT1-receptor effector complex and potentially avoid ACE and aldosterone escape mechanisms Van de Wal et al., 2005). Because RAAS plays an important role in insulin resistance, we hypothesized that blockade of the RAAS with the combined treatment of aliskiren and olmesartan for 8 weeks would improve glucose homeostasis, glomerular filtration rate along with renal variables in streptozotocin induced diabetic rats (Schiffrin et al., 2007).

2. Materials and methods

2.1. Materials

Aliskiren (10 mg/kg s.c. via osmotic drug delivery) procured from Novartis, R & D Base, Switzerland, Basel; olmesartan procured from Ranbaxy Lab. Ltd., India (6 mg/kg/day); Quantichrom creatinine Assay Kit (DICT-500), Rat Albumin ELISA by (ICL Cat E-25L Lot #14), Ultra sensitive Rat Insulin ELISA Kit (by Crystal Chem Inc. Catalog # 90060), Rat Erythropoietin ELISA Kit (Cusabiotech Co. Catalog No. CSB-E07323r), Rat beta-2 microglobulin (BMG, Cusabiotech Co. Catalog No. CSB-E11298r), Rat Cystatin C (Cys-C) ELISA Kit (Cusabiotech Co. Catalog No. CSB-E08385r), Rat transforming growth factor β1 (TGF-β1) ELISA Kit (Cusabiotech Co. Catalog No. CSB-E04727r), Rat TNF-α ELISA Kit (Raybio Cat #ELR-TNFalpha-001), Human/Mouse/rat Adiponectin Enzyme Immunoassay Kit (Raybio Cat#EIA-ACRP-1), Rat VEGF ELISA Kit (Raybio Cat #ELR-VEGF-001).

2.2. Animals

Healthy albino rats of Wistar strain were kept for breeding. To induce NIDDM, STZ (Sigma Chemicals, USA) (90 mg/kg) was administered i.p. to a group of 2 days old pups. Another group of pups received only saline. The pups were weaned for 21 days, and 6 weeks after the injection of STZ, the animals were checked for fasting glucose level (FPG) ≥ 160 mg/dl were considered as diabetic. Pups that receive saline were considered as control animals, after which they were grouped so that the blood glucose levels were uniform among the groups. All rats were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet and water (ad libitum). All experimental procedures were conducted according to the Institutional Animal Ethical Committee (Protocol No. DIPSAR/IAEC/2009/01) and CPCSEA guidelines.

2.3. Eight week chronic daily dosing study

After 6 weeks, the animals were assigned to receive vehicle, aliskiren (10 mg/kg/day; s.c. via osmotic mini pump), olmesartan (6 mg/kg/day; orally) and combination of both aliskiren and olmesartan once daily for 8 weeks. On the morning after final administration, blood samples were collected under fasting conditions and body weight was measured; and the kidney was isolated and was immersed and fixed in phosphate-buffered 10% formalin solution to prepare a paraffin section.

2.4. GLUT-2 expressions in liver and GLUT-4 expressions in soleus muscle

For determination of glucose transporter-2 (GLUT-2) protein expressions in liver and glucose transporter-4 (GLUT-4) protein expressions in skeletal muscle; each sample prepared was mixed with 1% sodium dodecyl sulfate and 50 mM dithiothreitol, and the mixture was subjected to electrophoresis with 10% polyacrylamide gel. The separated proteins on the gel were electrotransferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk solution including 0.05% poly (oxyethylene) sorbitan monolaurate (Tween 20) overnight at 4°C, the membrane was reacted with anti-GLUT-2 antibody (Abcam, UK) and anti-GLUT-4 antibody (Abcam, UK) for 2 h. Subsequently, it was incubated with horseradish peroxidase conjugated IgG (diluted 1:2000) [Jackson ImmunoResearch Laboratories, USA] for 2 h at room temperature. The blots were detected with chemiluminescence reagents (Western blot) (Yoshihiko et al., 2007).

2.5. Measurement of hemodynamic parameters in normal rats

Hemodynamic parameters were measured by using tail cuff apparatus (Kent Scientific, USA).

2.6. Measurement of renal function and biochemical parameters

Biochemical markers including blood glucose, plasma insulin, serum albumin, total plasma proteins; glomerular proteins viz β-2 microglobulin, serum cystatin C and serum creatinin, for the estimation of GFR; transforming growth factor (TGF-β1) and other molecular markers specifically adiponectin and erythropoietin and inflammatory markers TNF-α, NO and VEGF.

2.7. Histopathology

Kidney sections were stained with either periodic acid-Schiff’s reagent or Masson’s modified trichrome to assess glomerulosclerosis and demonstrate collagenous tubulointerstitial matrix, respectively (Kelly et al., 2007).

2.7.1. Glomerulosclerotic index

In 4 μm kidney sections stained with periodic acid-Schiff’s reagent, 150 glomeruli from each animal were examined in a masked protocol. The extent of sclerosis in each glomerulus was subjectively graded on a scale of 0–4, with the following grades: grade 0 normal, grade 1 sclerotic area <25% (minimal), grade 2 sclerotic area 25–50% (moderate), grade 3 sclerotic area 50–75% (moderate to severe) and grade 4 sclerotic area 75–100% (severe). A glomerulosclerotic index was then calculated using the formula:

\[ GSI = \sum_{i=0}^{4} Fi \]  

(1)

where GSI is glomerulosclerotic index, \( Fi \) is the % of glomeruli in the rat with a given score (i).

2.7.2. Quantitation of matrix deposition

The accumulation of matrix within the tubulointerstitial was assessed on Masson's trichrome stained sections. An area of blue on a trichrome-stained section was selected for its color range with the proportional area of tissue with this range of color was then semi-quantified. The proportional area stained blue (matrix) was then determined and semi-quantified and graded by similar method as performed for glomerulosclerotic index.
2.8. DNA fragmentation assay

For detection and localization of apoptosis in pancreas, we used the technique of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit, Biovision, USA). Briefly, sections were deparaffinized, hydrated, and digested with proteinase K (20 μg/ml), and then added biotinylated dUTP to the 3’ end of DNA fragments by incubating sections in 0.05 mol/l Tris–HCl buffer (pH 7.6) with 0.03 U/μl TdT and 0.04 nmol/μl biotin-11-dUTP at 37°C for 1 h. The sections were rinsed in PBS. Endogenous peroxidase was blocked with 0.3% H2O2 in distilled H2O. The sections were rinsed with PBS and covers with 2% blocking solution in 0.1 mol/l sodium maleate to reduce background staining. The sections were then incubated with avidin-peroxidase complexes in PBS (1:50) for 30 min and rinsed with PBS (3 × 5 min). Peroxidase activity was visualized with 3,3’-diaminobenzidine until the brown product was clearly visible. The sections were then counterstained with methyl green. The positive apoptotic cells were the cells with brown nucleus (Matsuno et al., 1997).

2.9. Statistical analysis

All data are expressed as the mean ± S.EM. The differences in all parameters were analyzed by a one-way analysis of variance (ANOVA) followed by a Dunnett’s Multiple Comparison Test using sigma plot. A change was considered statistically significant if p < 0.05.

3. Results

3.1. Effect of aliskiren and olmesartan administration on body weight and blood glucose in monotherapy and in combination

Before the treatment, there were no significant differences of baseline body weight of the rats (Fig. 1 and Table 1). In monotherapy and combination treatment, significant increase in body weight (224.2 ± 4.1, p < 0.05; 216.7 g ± 4.77, p < 0.01 and 229.2 ± 4.5, p < 0.001) was observed, as compared with diabetic groups (198.3 g ± 3.1) after 8 weeks study (Table 1).

Before treatment, there was significantly higher fasting plasma glucose (p < 0.001) in diabetic and treated groups when compared with normal (Table 1). After 8 weeks, groups treated with individual and combination of aliskiren and olmesartan showed significant reduction of blood glucose (139.7 ± 5.05, p < 0.01; 144.7 ± 2.09, p < 0.05; 117.5 ± 4.03, p < 0.001) versus diabetic group (164.2 ± 6.5) (Table 1).

3.2. Effect of aliskiren and olmesartan monotherapy and combination treatment on liver GLUT-2 and muscle GLUT-4 expression

In diabetic rats, GLUT-2 expressions in liver and GLUT-4 expression in soleus muscle was reduced; whereas individual and combination treatment of aliskiren and olmesartan rats to the diabetic rats showed improvement in both GLUT-2 and GLUT-4 expressions (Fig. 2).

3.3. Biochemical markers

3.3.1. Plasma insulin

Aliskiren and olmesartan significantly increased plasma insulin (p < 0.01) to almost equivalent levels in the treated rats as compared with diabetic groups after 8 weeks (Table 2), whereas in the treatment with the combination the increase in plasma insulin levels were highly significant (p < 0.001).

3.3.2. Total proteins and albumin in plasma

Albumin and total proteins levels were found to increase significantly in plasma in the STZ treated diabetic rats. The amplification of protein levels was highly significant in both monotherapy and combination (p < 0.01 and p < 0.001, respectively). At the same time, the increase in plasma level of albumin was not statistically
significant when individual drugs were given, but became significant when given in combination \( (p < 0.05) \) (Table 2).

### Hemodynamic parameters

No significant changes were obtained in the changes in hemodynamic parameters on administration with individual and combination treatment with aliskiren and olmesartan (Fig. 1).

### Glomerular proteins and glomerular filtration rate (GFR)

#### 3.4.1. Serum creatinine

Diabetic control rats had elevated serum creatinine (Normal: 1.211 mg/dl ± 0.21; diabetic: 3.59 mg/dl), which was significantly reduced in drug treatment both with monotherapy of olmesartan \( (p < 0.05) \) and aliskiren \( (p < 0.01) \) and also in combination \( (p < 0.01) \) after 8 weeks chronic dosing (Table 3).

#### 3.4.2. Serum cystatin-C

Serum cystatin C has been increased in the diabetic rats. Treatment with the predetermined doses of aliskiren and olmesartan individually to the Streptozotocin induced diabetic rats decreased the cystatin C levels significantly in the serum \( (p < 0.05) \). However, when the combination of both the drugs was administered, the levels of serum cystatin C were decreased with high degree of statistical significance (Table 3).

#### 3.4.3. Beta-2 microglobulin

The treatment with aliskiren and olmesartan individually led to significant decrease in the serum beta-2 microglobulin concentrations \( (p < 0.05) \) in comparison with the diabetic control group (Table 3). At the same time, when both the drugs were given in combination the decrease in serum concentrations was observed with higher significance \( (p < 0.01) \).

#### 3.4.4. TNF-alpha

Further, significant difference in the mean values of TNF-\( \alpha \) observed in plasma between normal control and diabetic group \( (p < 0.01) \) when compared with normal rats (Table 4). After the 8-week treatment with aliskiren and olmesartan independently, the levels of TNF-\( \alpha \) significantly decreased when compared with the levels in diabetic rat \( (p < 0.05) \); Table 4). However, the decrease was highly significant statistically when given in combination \( (p < 0.01); \) Table 4).

#### 3.4.5. Nitric Oxide

Nitric Oxide levels were significantly increased in the serum of diabetic rats \( (p < 0.01) \). On treatment with aliskiren and olmesartan separately and in conjunction, nitric oxide levels were found to decrease in comparison with the diabetic group, though the decrease is found to be non-significant in either case (Table 4).

---

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<td>Normal</td>
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<td>231.7 ± 4.6***</td>
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<tr>
<td>Diabetic</td>
<td>131.7 ± 4.4</td>
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<tr>
<td>Olmesartan (6 mg/kg)</td>
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<td>Aliskiren (10 mg/kg)</td>
<td>141.7 ± 8.3</td>
<td>224.2 ± 4.11**</td>
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<td>Aliskiren + olmesartan</td>
<td>137.5 ± 11.0</td>
<td>229.2 ± 4.45***</td>
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</table>

The values are the means ± S.E.M. from eight animals in each group.

\[ *** \; p < 0.001 \text{ vs. normal group.} \]

\[ ** \; p < 0.01 \text{ vs. diabetic group.} \]

\[ * \; p < 0.05 \text{ vs. diabetic group.} \]

\[ ** \; p < 0.01 \text{ vs. diabetic group.} \]

\[ *** \; p < 0.001 \text{ vs. diabetic group.} \]

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**Fig. 2.** Effects of administration of aliskiren, olmesartan and in combination on GLUT–4 expression in the muscles and GLUT–2 expression in the liver of diabetic rats after chronic drug administration.
Table 2: Effect of aliskiren, olmesartan and combination on plasma insulin, total proteins and albumin.

<table>
<thead>
<tr>
<th></th>
<th>Plasma insulin (ng/ml)</th>
<th>Total proteins (mg/ml)</th>
<th>Albumin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17.83 ± 3.2**</td>
<td>2.144 ± 0.076**</td>
<td>1.26 ± 0.328**</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3.265 ± 0.36</td>
<td>1.368 ± 0.17</td>
<td>0.14 ± 0.501</td>
</tr>
<tr>
<td>Olmesartan (6 mg/kg)</td>
<td>12.74 ± 1.48**</td>
<td>1.935 ± 0.063**</td>
<td>0.74 ± 0.101</td>
</tr>
<tr>
<td>Aliskiren (10 mg/kg)</td>
<td>12.74 ± 1.77**</td>
<td>2.029 ± 0.1**</td>
<td>0.817 ± 0.17**</td>
</tr>
<tr>
<td>Aliskiren + olmesartan</td>
<td>15.07 ± 1.52**</td>
<td>2.123 ± 0.11***</td>
<td>1.18 ± 28.1**</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group.

- *p < 0.05 vs. diabetic group.
- **p < 0.01 vs. diabetic group.
- ***p < 0.001 vs. diabetic group.

Table 3: Effect of aliskiren, olmesartan and combination on glomerular proteins and serum creatinine.

<table>
<thead>
<tr>
<th></th>
<th>Serum cystatin C (ng/ml)</th>
<th>BMG (µg/ml)</th>
<th>Serum creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.2298 ± 0.026**</td>
<td>0.4419 ± 0.048**</td>
<td>1.211 ± 0.21**</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.644 ± 0.033</td>
<td>1.362 ± 0.12</td>
<td>3.589 ± 0.18</td>
</tr>
<tr>
<td>Olmesartan (6 mg/kg)</td>
<td>0.4617 ± 0.048**</td>
<td>0.6529 ± 0.21</td>
<td>2.063 ± 0.41</td>
</tr>
<tr>
<td>Aliskiren (10 mg/kg)</td>
<td>0.4628 ± 0.058**</td>
<td>0.6709 ± 0.18</td>
<td>1.753 ± 0.27**</td>
</tr>
<tr>
<td>Aliskiren + olmesartan</td>
<td>0.2538 ± 0.036**</td>
<td>0.542 ± 0.10**</td>
<td>1.613 ± 0.37**</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group.

- *p < 0.05 vs. diabetic group.
- **p < 0.01 vs. diabetic group.
- ***p < 0.001 vs. diabetic group.

Table 4: Effect of aliskiren, olmesartan and combination on inflammatory and growth factors.

<table>
<thead>
<tr>
<th></th>
<th>TNF-alpha (pg/ml)</th>
<th>NO (nmol/µl)</th>
<th>TGF-beta (pg/ml)</th>
<th>VEGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.0176 ± 0.00545**</td>
<td>0.0176 ± 0.00545**</td>
<td>3.361 ± 0.98**</td>
<td>48.5 ± 3.05**</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.427 ± 0.2198</td>
<td>0.427 ± 0.2198</td>
<td>11.3 ± 1.348</td>
<td>74.08 ± 5.34</td>
</tr>
<tr>
<td>Olmesartan (6 mg/kg)</td>
<td>0.1151 ± 0.031**</td>
<td>0.34 ± 0.097</td>
<td>5.963 ± 0.896**</td>
<td>66.3 ± 1.14</td>
</tr>
<tr>
<td>Aliskiren (10 mg/kg)</td>
<td>0.08701 ± 0.020**</td>
<td>0.087 ± 0.020**</td>
<td>6.673 ± 0.44**</td>
<td>64.88 ± 1.16</td>
</tr>
<tr>
<td>Aliskiren + olmesartan</td>
<td>0.05565 ± 0.011**</td>
<td>0.29 ± 0.086</td>
<td>4.376 ± 0.89**</td>
<td>58.48 ± 2.35**</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group.

- *p < 0.05 vs. diabetic group.
- **p < 0.01 vs. diabetic group.
- ***p < 0.001 vs. diabetic group.

3.5. Growth factor

3.5.1. Transforming growth factor-b1 (TGF-b1)

TGF-b1, a key participant in the development of kidney sclerosis significantly differs in diabetic and normal control group (p < 0.001) (Table 4). The plasma concentration of TGF-b1 in aliskiren and olmesartan treated rats was decreased significantly (p < 0.05 and p < 0.01), respectively when compared with the diabetic group after 8-weeks (Table 4). However, when both the drugs were administered in combination the decrease observed was found to be highly statistically significant (p < 0.01).

3.5.2. Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a critical component in the tissue growth and organ repair processes of angiogenesis and vasculogenesis. VEGF was significantly lower in the normal control group than in the diabetic group (Table 4) (p < 0.001). The aliskiren and olmesartan treatment when given alone reduced the plasma concentration of VEGF which is not statistically significant (Table 4) in comparison with the diabetic group. However, the combined administration of drugs significantly decreased the concentration of VEGF in plasma.

3.6. Molecular markers

3.6.1. Adiponectin

Adiponectin measured as a surrogate marker for inflammation was significantly lower in diabetic group from normal control group (p < 0.01). On individual treatment with aliskiren and olmesartan, adiponectin levels were increased but the augmentation was not found to be statistically significant (Table 5). But, the combined treatment of both the drugs led to the significant improvement in adiponectin levels.

3.6.2. Erythropoietin

The erythropoietin concentration in plasma was found to increase significantly when treated with aliskiren alone (p < 0.05), though the increase was not statistically significant when the diabetic rats were administered olmesartan alone. However, when the combination of both was administered the erythropoietin levels were found to increase with higher level of significance (p < 0.01) (Table 5).

3.7. Histopathology

In normal rats, the kidney cortex appeared normal (Fig. 3). In contrast, in diabetic rats most of glomeruli exhibited thickened glomerular basement membrane, capillary occlusion, and mesangial expansion. In addition, many cortical tubules were vacuolated. In diabetic rats treated with aliskiren and olmesartan, glomerular pathology was improved (Fig. 3). However, when the diabetic rats were treated with the combination of aliskiren and olmesartan brought the pathological parameters to almost normal. The Glomerular loops developed thin basement membranes (GBM) and the mesangial matrix was mildly increased. Tubular basement membranes (TBM) were within normal limits.
3.7.1. Glomerulosclerotic index (GSI)
Diabetes was associated with an increase in GSI. Compared to diabetic control rats, GSI was lowered in diabetic rats treated with olmesartan and aliskiren alone and in combination (Fig. 4).

3.7.2. Quantitation of matrix deposition
In the tubulo-interstitium of kidney cortex or medulla, increased collagen and inflammatory cells were observed in diabetic control rats compared to control rats. Compared to diabetic control rats, interstitial fibrosis was lowered in treatment with aliskiren and olmesartan alone and in combination (Fig. 4).

3.8. Apoptosis
There was an evident increase in the DNA fragmentation in diabetic kidney compared to the normal kidney (Fig. 3). Whereas the rat treated with aliskiren and olmesartan showed a clear decrease in the extent of DNA fragmentation, which was nearly normalized in treatment with combination of the two drugs (Fig. 3).

4. Discussion
The direct inhibition of renin is a logical target for pharmacologic suppression of the RAAS, because renin-mediated cleavage of angiotensinogen to form Angiotensin I is a rate-limiting first step in the RAAS pathway. Neonatal–STZ Wistar model is well characterized model of type 2 diabetes. Neonatal–STZ rats develop persistent diabetes rapidly after 6 weeks of age, and showed diabetes like

### Table 5
Effect of aliskiren, olmesartan and combination on molecular mediators.

<table>
<thead>
<tr>
<th></th>
<th>Adiponectin (µg/ml)</th>
<th>Erythropoetin (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.013 ± 0.21</td>
<td>0.6775 ± 0.026</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.4 ± 0.089</td>
<td>0.1283 ± 0.064</td>
</tr>
<tr>
<td>Olmesartan (6 mg/kg)</td>
<td>0.93 ± 0.18</td>
<td>0.2657 ± 0.047</td>
</tr>
<tr>
<td>Aliskiren (10 mg/kg)</td>
<td>1.34 ± 0.58</td>
<td>0.4641 ± 0.112</td>
</tr>
<tr>
<td>Aliskiren + olmesartan</td>
<td>2.678 ± 0.44</td>
<td>0.5455 ± 0.097</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group.

p < 0.05 vs. diabetic group.

p < 0.01 vs. diabetic group.

p < 0.001 vs. diabetic group.

Fig. 3. Effect of 8 weeks dosing of combination and monotherapy of aliskiren and olmesartan on histopathologic changes and cellular apoptosis (using TUNNEL-positive cells) in the kidney of STZ diabetic rats. Periodic acid-Schiff's reagent-stained, Masson's trichrome-stained and methylene green stained (for TUNNEL-positive) sections are represented for (A–C) normal rats, (D–F) vehicle-treated diabetic rats, (G–I) aliskiren (10 mg/kg), (J–L) olmesartan (6 mg/kg/day) and combination of aliskiren and olmesartan (M–O)-treated diabetic rats, respectively. Original magnification 400× (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
symptoms such as lack of insulin release in response to glucose, glucose intolerance, raised glycosylated hemoglobin, and depletion of pancreatic insulin store (Weir et al., 1981; Daniel, 1991; Masiello et al., 1998). Further, when rendered diabetic with streptozotocin (STZ), they develop renal damage considered analogous to that seen in human Diabetic Nephropathy (Kelly et al., 1998). At the same time, it has been said that diabetes induced by STZ injection significantly down regulates the protein expression of ACE-2 and Ang-(1–7) mas R, which eventually causes renal hypertrophy, tissue injury, and apoptosis via the upregulated phosphorylation of ERK1/2, p38 MAPK, and JNK protein expression (Lakshmanan et al., 2011).

Blood pressure-lowering efficacy of aliskiren in hypertensive patients is also well known and authenticated by various clinical trials (Stanton et al., 2003; Oparil et al., 2007; O’Brien et al., 2007). Pilz et al. also demonstrated a protective effect of aliskiren for both heart and kidney injuries in double transgenic rats for human renin and angiotensinogen genes (Pilz et al., 2005). However, in the recent clinical study on aliskiren in the Evaluation of Proteinuria in Diabetes (AVOID), aliskiren, combined with losartan, showed a significant antiproteinuric effect that was independent of its blood pressure-lowering effect in patients with hypertension and diabetic nephropathy (Parving et al., 2008). Kelly et al. illustrated that aliskiren had a similar efficacy to ACE inhibitor for reduction of urinary albumin and glomerulosclerosis in diabetic transgenic (mRen-2)27 rats (Kelly et al., 2007). Together, these results suggest a beneficial effect of renin inhibition in hypertensive and diabetic renal disease. However, it does not eliminate the need of multidrug regimens in the treatment of hypertension and renal complications in diabetes.

Thus, present work highlights the combination of aliskiren and olmesartan as a promising therapeutic strategy for renal complications in diabetes. The selection of doses of the drugs have been done on the basis of previous studies (Pilz et al., 2005; Pugsley, 2005).

In pursuit to that, the diabetic rats in the present study experienced approximately 81% decrease in pancreatic insulin content, which was significantly improved with both combination and monotherapy. However, in combination the insulin levels were improved with higher degree of significance. At the same time, aliskiren and olmesartan treatment alone significantly reduced blood glucose and additive effects were observed when both the drugs were administered in combination. Aliskiren showed increase in body weight as compared to diabetic rats, which was in contrast to the results of aliskiren treatment on C57BL/6j mice (Stucchi et al., 2009). In addition, rats treated with olmesartan alone also showed weight gain. Highly significant effects were observed in combination treatment of aliskiren and olmesartan, as with combination treatment of aliskiren and other ARBs (Nyathani et al., 2011).

The glucotransporter isoforms GLUT-2 and GLUT-4 play central roles in the complex pathways mediating whole body glucose disposal, in which dysregulation of their controlling mechanism can result in the pathophysiologic states associated with diabetes (Maria et al., 2001). Aliskiren and olmesartan treated diabetic rats improved liver and muscle glucotransporter expression levels when administered single handedly. More dense bands were observed in combination when compared with monotherapy. These results qualify the improved insulin sensitivity not only for individual drugs but also when the drugs were used together. The plausible mechanism for this could be the vasodilatory action by both the drugs which become synergistic when given in combination, thereby increasing skeletal muscle blood flow to improve insulin sensitivity and glucose homeostasis (Azizi et al., 2004).

Furthermore, proteinuria, insulin resistance and formation of reactive oxygen species (ROS) are also associated with loss of adiponectin level in diabetic rats. STZ induced diabetic rats showed almost 87% decrease in adiponectin levels as compared with normal rats, which was found to be improved with both combination and monotherapy. However, the combination treatment improved the levels to a significant degree. Adiponectin can improve both glucose metabolism and insulin resistance via the AMP-activated protein kinase (AMPK) signaling pathway both in liver and muscle and lower the blood glucose concentrations in vivo (Scherer et al., 1998; Yamashita et al., 2002). Studies have shown that phosphorylation of AMPK-α (Thr172) is decreased in diabetic rats. The decreased plasma adiponectin levels may partly explain the decreased phosphorylation of AMPK-α (Thr172) in diabetic rats. AMPK phosphorylation increases glucose transport by stimulating the translocation of GLUT-4. This finding of the improvement of adiponectin levels in the diabetic treated rats can further be confirmed by the significant increase in body weight of the animals when given combined treatment, although the increase in adiponectin levels was not significant when treated with the drugs individually. Hence, RAAS blockade increased adiponectin concentrations with a consequent improvement in insulin sensitivity and secretion. Increase in adiponectin levels in both monotherapy and combination study and partial agonistic nature of olmesartan to PPAR receptors in adipocytes, macrophages, immune cells and cells from the vascular wall (Gelman et al., 2007) has also been supposed to describe the anti-inflammatory actions of the drugs used. The proposed exposition was justified by the significant reduction of levels of TNF-α in combination when compared with individual treatment groups and thereby inhibiting apoptosis notably. These drugs by inhibiting NAD (P) H oxidase, and consequently reducing oxidative stress have been shown to exert protective, anti-diabetogenic effects on pancreatic and renal cells (Stergren, 2007).

In this study, only a single dose of each drug was chosen, aiming to achieve similar blood pressure reductions in both treatment
groups. Also, the additive or synergistic effects of such combinations have been shown to be more evident at low doses (which are the usual dose) than at high doses (Maillard et al., 2002; Morgan et al., 2002; Mann and Deswal, 2003).

Aliskiren and olmesartan monotherapy reduced BP by 13.4% and 9.5%, respectively whereas combination showed reduction of blood pressure by 15%. From this result, we can put the finding that combination did not show any significant effect on blood pressure. These findings are consistent with preclinical (Nyathani et al., 2011) and clinical (Parving et al., 2008; Moriyama et al., 2011; Fossa et al., 1994) studies using different combinations of direct renin inhibitor aliskiren and angiotensin receptor blocker.

In patients with kidney disease blood pressure apart from adiponectin (Nakamaki et al., 2011) correlates closely with the magnitude of proteinuria. In patients with diabetic nephropathy, ACE inhibitor and ARB therapy reduces proteinuria by a range of intra-renal effects including modulation of intra-glomerular pressure, permeability and matrix protein production (Kelly et al., 2007). Moreover, studies indicate that albuminuria is a major renal and cardiovascular risk factor in patients with diabetes (De Zeeuw et al., 2004a, 2004b; Atkins et al., 2005). Interventions that have attenuated the progression of diabetic nephropathy have been associated with a reduction in urinary protein excretion (Parving et al., 2008), and thus, renoprotective therapy should aim to achieve the maximal anti-albuminuric effect (De Jong et al., 1999; Eijkelkamp et al., 2007). However, a recent study proposed that in twenty diabetic patients with macroalbuminuria, large quantities of protein fragments, that are not routinely measured clinically, were present (Grieve et al., 2001). Also, lower serum albumin concentration imposes a greater increase in relative risk of mortality among populations as serum values of albumin are not readily impacted by minor variations by the body's natural mechanism of maintaining homeostasis in albumin metabolism (Kaysen and Don, 2010). Albumin is also a major source of sulphoryl groups and these thiols scavenge free oxygen and nitrogen radicals and other toxins (Roy et al., 1998). This may be an important function in the activation of systemic inflammatory response thereby increasing vascular permeability. Hence, we choose serum albumin as an important marker for identification of progression of renal damage. In the present investigation, combination therapy increased serum albumin significantly bringing the values to near normal as compared to the individual treatments despite the negligible differences in BP between the combination and monotherapy. Moreover, no correlation between serum albumin and cystatin C are found, thereby shifting the efferent arteriole raises intra-glomerular pressure. Further, it induces the expression of fibrotic growth factors that is TGF-β1 and VEGF via MAPK p42/p44, thereby upregulating molecules like fibronectin, collagen-1 and plasminogen-activator inhibitor-1 (PAI-1) (Chen et al., 2009; Huang et al., 2006, 2007) leading to glomerulosclerosis (Johnston et al., 1998; Brenner, 1983; Anderson, 1997). In the present study, both olmesartan and aliskiren reduced glomerulosclerosis both in combination and individual treatment to similar degrees, despite differing effects on systemic blood pressure which is further supported by significant reduction of TGF-β1 and VEGF. VEGF in glomeruli directly has been associated with glomerular hypertrophy and increased production of alpha3 (IV) collagen, which is an integral component of the glomerular basement membrane (GBM) thereby leading to sclerosis. In the present study, the combination treatment also attenuated tubulointerstitial fibrosis, another important predictor of renal dysfunction (Kelly et al., 2007) to a similar extent as that of individual treatment.

In addition, anemia has been associated with an accelerated decline in renal function in some of the patient groups. Decreased tissue oxygen delivery caused by anemia stimulates the renin angiotensin aldosterone system and contributes to renal vasoconstriction. These factors can further exacerbate proteinuria, which may worsen renal function. In patients with type 2 diabetes, anemia has been shown to be an independent risk factor for progression of renal disease (Fisher, 2003). The significant improvement of erythropoietin levels in combination treatment as compared to monotherapy showed extended ambit produced by the combination treatment in delaying progression to renal complications. Though the meliorism was achieved with both the drugs when given alone but only aliskiren ameliorated the erythropoietin levels with significance. Enhanced inflammatory cytokines and reactive oxygen species could play a role in reducing the responsiveness of precursor cells in the bone marrow to erythropoietin (Casadevall, 1995 and Macdougall and Cooper, 2002).

Further, to substantiate the renal variables, measuring GFR has been widely accepted as the best overall index of kidney function. In clinical practice, an approximation of GFR can often be obtained from plasma/urine creatinine concentration alone albeit with limited accuracy (Perrone et al., 1992). Pure and reliable urinary samples have been recognized as very challenging to obtain from experimental animals, especially from small rodents (Kurien et al., 2004). Moreover, serum creatinine is particularly insensitive for identifying chronic kidney disease in its early to middle stages and in certain patient groups (e.g. children, females, elderly) (Davìd, 2005). It has been considered relatively specific but not very sensitive since its levels significantly increase only when more than 50% of the GFR is reduced. Measurement of cystatin C or beta2microglobulin (B2M) concentrations has been found to be advantageous compared with measurements of serum creatinine concentration for the detection of an impaired GFR (Filler et al., 1997). Cystatin C and B2M are low-molecular weight endogenous proteins freely filtered by the glomerulus and proximal tubule, respectively (Kezama et al., 2002). It is already shown that serum cystatin C levels rise earlier and more rapidly than those of serum creatinine as GFR decreases (Tengstad et al., 1996), reflecting the greater sensitivity and hence diagnostic accuracy of cystatin C as a predictor of GFR in type 2 diabetic patients. Further, B2M has been shown to be unstable in acidic and infected alkaline urine, hence may degrade during the time urine is held in the bladder; therefore, its absence cannot be relied upon to exclude the presence of a tubular proteinuria (Lapsley et al., 1991). Therefore, we preferred to perform the analysis of the filtration markers in serum, as also their serum concentrations are less dependent on extra renal factors than in the case of serum creatinine (Morin et al., 2007). In the nephrotic syndrome, the increased excretion of low molecular weight plasma protein has been explained by accompanying tubular malfunction, but in renal failure, major factor includes the overwhelming reabsorptive capacity in the less affected nephrons. Studies have indicated that patients excreting increased
quantities of low molecular weight proteins were concurrently also excreting increased amounts of albumin. Thus, increased albumin excretion does not necessarily indicate glomerular disease. In the present study, it was obvious that combination and monotherapy both improved the creatinine clearance, as there was significant decrease in serum creatinine values. At the same time, serum levels of cystatin C and BMG were significantly decreased when compared with diabetic rats after chronic administration of combination and monotherapy. These findings were consistent with the study done by Byung et al. (2007).

5. Conclusion

Besides life-style modifications and classical pharmacological strategies using various anti-diabetic agents, drugs that inhibit RAAS activity might be considered as a valuable alternative to delay the progression of type 2 diabetes mellitus to its renal complications, especially in patients with arterial hypertension who are well recognized at risk to develop the disease. The results of the present study showed that in streptozotocin induced diabetic rats, the RAAS blockade induced by a low dose of an AT1R antagonist viz. olmesartan can be enhanced by inhibiting renin activity, through co-administration of a potent, long-lasting renin inhibitor, aliskiren which further prevents the progression of the diabetes to its complications by improving glucose sensitivity and renal parameters without affecting much of hemodynamics of the rats.

The apprehension that aliskiren might not provide sufficient exposure to inhibit both the basal renin activity as well as the released renin due to the feedback activation of the RAAS because of its low absolute bioavailability (approximately 2%) could not be expressed from the results of parameters studied in the combination treatment. However, bantam values so obtained with individual drug treatment could be due to increased expression of prorenin. It is also believed that much of the antihypertensive efficacy of renin inhibitors emanate from inhibition of tissue rather than circulating renin, the efficacy of aliskiren in the extravascular renin compartment needs to be explored. Hence, it is possible that renin inhibitors preferentially inhibit tissue renin which will provide a more effective and sustained RAAS inhibition. Further, signaling pathways activated by (pro) renin binding to its receptor which are expected to play a role in disease progression needs to be evaluated. In addition, the results obtained in the study further support clinical studies at different doses of these compounds to investigate the long-term effects of renin inhibitor–AT1R antagonist combinations in various clinical contexts, including hypertension and chronic proteinuric nephropathies.

Conflicts of interest

There are no conflicts of interest.

Acknowledgements

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Birkenhäger, W.H., Staessen, J.A., 2007. Dual inhibition of the renin system by aliskiren and valsartan. This study was supported by University Grants commission, Delhi under Major Research (F. No. 34/135/2008). We also thank Tracy Maden, Novartis, Switzerland for providing generous gift of aliskiren. S. Gandhi et al. / European Journal of Pharmaceutical Sciences 46 (2012) 32–42
Aliskiren, a human renin inhibitor, ameliorates cardiac and renal damage in double-transgenic rats. Hypertension 46, 569–576.


Aliskiren improves insulin resistance and ameliorates diabetic renal vascular complications in STZ-induced diabetic rats

Sonia Gandhi, BP Srinivasan and Atul Sureshrao Akarte

Abstract
Aliskiren, a direct renin inhibitor (DRI), has therapeutic effects in patients with hypertension and associated complications, but its potential mechanism in diabetic nephropathy is lacking. The effects of aliskiren in Streptozotocin (STZ)-induced renal complication in diabetic rats were investigated. Aliskiren treatment for eight weeks at a dose of 10 mg/kg/day, via osmotic mini-pump, induced improvement in blood glucose levels, systolic blood pressure (BP) and serum creatinine. Improvement of insulin resistance by aliskiren was confirmed by increased glucose translocation in liver and muscle and hence insulin levels. The treated group also showed improvement in glomerulosclerosis and tubulointerstitial injury. Aliskiren treatment also improved albumin levels in plasma, suppressed profibrotic and proinflammatory cytokine synthesis viz TNF-α and TGF-β and angiogenesis by a decrease in VEGF. In addition, the level of total proteins and GFR via cystatin c and beta-2microglobulin along with adiponectin and erythropoietin were also improved. These results suggest that the beneficial organ protective effect of aliskiren is mediated by improvement in insulin resistance as well as a direct anti-fibrotic effect in the target organ in STZ-induced diabetic rats with a slight effect on blood pressure. Aliskiren may be a useful therapeutic agent in the treatment of type 2 diabetes and diabetic nephropathy.

Keywords
Direct renin inhibitor, aliskiren, diabetic nephropathy, glomerulosclerosis, glomerular filtration rate markers, insulin resistance

Introduction
Dysregulation of the renin-angiotensin-aldosterone system (RAAS) has an important role in the development of target organ damage in diabetes mellitus.1–3 Therefore, drugs targeting RAAS suppression have been most widely used for preventing or delaying target organ damage.1 Most RAAS blockers, however, are not completely effective in preventing or reversing diabetic complications, which could be due to the stimulation of renin that results from the negative feedback loop associated with decreased angiotensin II activation. Thus, direct renin inhibition appears to be more rational to create a complete and effective blockade of RAAS activity without a rebound increase in plasma renin activity (PRA).4,5 Aliskiren is the first drug of a new class of agents known as renin inhibitors, which directly inhibit renin in order to decrease PRA.6 Unlike previous renin inhibitors, aliskiren has better oral bioavailability and an extended half-life.7 The most recent reports for aliskiren treatment revealed effective organ protection related to an anti-hypertensive effect.8–11 Previous studies showed protective renal and cardiac benefits of aliskiren treatment by monotherapy or combination therapy with other RAAS blockades in both human and animal studies. But there is a lack of evidence on the effects of aliskiren on insulin resistance and renal variables along with a decline in the extent of apoptosis in the kidney in delaying the progression of target organ injury in a type 2 diabetic animal model. Recent hypertension trials have reported a lower incidence of diabetes mellitus among patients treated with RAAS inhibitor when compared with other classes of anti-hypertensive medications.12,13 Although most of the patients from those studies presented with hypertension or congestive heart failure, a 22% relative risk reduction for new-onset diabetes mellitus was demonstrated in a meta-analysis of randomized controlled trials.14 In spite of these firm clinical
data, the anti-diabetic mechanism of RAAS blockade ameliorating the diabetic complications is yet to be resolved. Multiple mechanisms such as improving insulin signaling pathways, insulin secretion by pancreatic beta cells and modulation of adipocytokines in the adipose tissues have been suggested. In the present study, we investigated mechanisms behind the beneficial effect of aliskiren on diabetic microvascular renal complications in type 2 diabetic rats. Since RAAS plays an important role in insulin resistance, we also examined the effect of aliskiren on insulin resistance and glucose translocation in liver and skeletal muscle.

Materials and methods

Materials

Aliskiren (10 mg/kg/day subcutaneously (s.c.) via osmotic drug delivery) procured from Novartis, R & D Base, Switzerland, Basel, Rat Albumin ELISA by (ICL Cat E-25L Lot #14), Ultra sensitive Rat Insulin ELISA Kit (by Crystal Chem. Inc Catalog # 90060), Rat Erythropoietin ELISA Kit (CUSABIO Technology Co. Catalog no. CSB-E07323r), Rat beta-2 microglobulin (BMG, Cusabio Technology Co. Catalog no. CSB-E11298r), Rat Cystatin C (Cys-C) ELISA Kit (CUSABIO Technology Co. Catalog no. CSB-E08385r), Rat transforming growth factor β1 (TGF-β1) ELISA kit (CUSABIO Technology Co. Catalog no. CSB-E04727r), Rat TNF-α ELISA Kit (Raybio Cat#ELR-TNFalpha-001), Human/Mouse/Rat Adiponectin Enzyme Immunoassay Kit (Raybio Cat#EIA-ACRP-1), Rat VEGF ELISA Kit (Raybio Cat#ELR-VEGF-001).

Animals

Healthy albino rats of Wistar strain were kept for breeding. To induce non-insulin dependent diabetes mellitus (NIDDM), Streptozotocin (STZ) (Sigma Chemicals, WA, USA) (90 mg/kg/day) was administered intraperitoneally (i.p.) to a group of two-day-old pups. Another group of pups received only saline. The pups were weaned for 21 days, and six weeks after the injection of STZ, the animals were checked for fasting plasma glucose level (FPG); levels ≥ 160 mg/dl were considered as diabetic. Pups that received saline were considered as control animals, after which they were grouped so that the blood glucose levels were uniform among the groups. All rats were housed under conventional conditions with controlled temperature, humidity and light (12-hour light-dark cycle) and were provided with a standard commercial diet and water (ad libitum). All experimental procedures were conducted according to the Institutional Animal Ethical Committee (protocol no. DIPSAR / IAEC / 2009/01) and Committee for the Purpose of Control and Supervision of Experiments on Animals (India) (CPCSEA) guidelines.

Eight-week chronic daily dosing study

After six weeks, the animals were assigned to receive vehicle or aliskiren (10 mg/kg/day s.c.) for eight weeks using an osmotic mini-pump. In the morning after final drug administration, blood samples were collected under fasting conditions and body weight was measured; and the kidney was isolated and fixed in phosphate-buffered 10% formalin solution to prepare a paraffin section.

Glucose transporter (GLUT)2 expressions in liver and GLUT4 expressions in soleus muscle

For determination of GLUT2 protein expressions in liver and GLUT4 protein expressions in skeletal muscle, each sample prepared was mixed with 1% sodium dodecyl sulfate and 50 mM dithiothreitol, and the mixture was subjected to electrophoresis with 10% polyacrylamide gel. The separated proteins on the gel were electrotransferred to a polyvinylidene difluoride membrane. After blocking with a 5% skim milk solution including 0.05% poly (oxyethylene) sorbitan monolaurate (Twee 20) overnight at 4°C, the membrane was reacted with anti-GLUT-2 antibody (Abcam, Cambridge, UK) and anti-GLUT-4 antibody (Abcam) for two hours. Subsequently, it was incubated with horseradish peroxidase conjugated IgG (diluted 1:2000) (Jackson Immunoresearch Laboratories, PA, USA) for two hours at room temperature. The blots were detected with chemiluminescence reagents (Western Blot).

Measurement of hemodynamic parameters in normal rats

Systolic, diastolic and mean blood pressure (BP) were measured by using a tail cuff apparatus (Kent Scientific, CT, USA).

Measurement of renal function and biochemical parameters

Biochemical markers were measured, including blood glucose, plasma insulin, serum albumin, total plasma proteins; glomerular proteins viz β-2 microglobulin, serum cystatin c and serum creatinine, for the estimation of glomerular filtration rate (GFR), transforming growth factor (TGF-β1) and other molecular markers, specifically adiponectin and erythropoietin, and inflammatory markers tumor necrosis factor alpha (TNF-α), nitric oxide (NO) and vascular endothelial growth factor (VEGF).

Histopathology

Kidney sections were stained with either periodic acid-Schiff’s reagent or Masson’s modified trichrome to
assess glomerulosclerosis and collagenous tubulointerstitial matrix, respectively.\textsuperscript{10}

**Glomerulosclerotic index**

In 4μm kidney sections stained with periodic acid-Schiff’s reagent, 150 glomeruli from each animal were examined in a masked protocol. The extent of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4,\textsuperscript{19} with the following grades: grade 0 normal, grade 1 sclerotic area <25% (minimal), grade 2 sclerotic area 25–50% (moderate), grade 3 sclerotic area 50–75% (moderate to severe) and grade 4 sclerotic area 75–100% (severe). A glomerulosclerotic index was then calculated using the formula:

\[
4 \quad \text{GSI} = \sum_{i=0}^{4} F_i (i)
\]

Where GSI is the glomerulosclerotic index, Fi is the percentage of glomeruli in the rat with a given score (i).

**Quantitation of matrix deposition**

The accumulation of matrix within the tubulointerstitial was assessed on Masson’s trichrome-stained sections. An area of blue on a trichrome-stained section was selected for its color range, and the proportional area of tissue with this range of color was then semi-quantified and graded by similar method for GSI and tubulointerstitial index (TIMI).

**DNA fragmentation assay**

For detection and localization of apoptosis in the pancreas, we used the technique of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Apo-BrDu-IHC\textsuperscript{TM} In Situ DNA Fragmentation Assay Kit, Biovison, CA, USA). Briefly, sections were deparaffinized, hydrated and digested with proteinase K (20 μg/ml), and then biotinylated dUTP was added to the 3’ end of DNA fragments by incubating sections in 0.05 mol/l Tris-HCl buffer (pH 7.6) with 0.03 U/μl TdT and 0.04 nmol/μl biotin-11-dUTP at 37°C for one hour. The sections were rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with 0.3% H₂O₂ in distilled H₂O. The sections were rinsed with PBS and covered with 2% blocking solution in 0.1 mol/l sodium maleate to reduce background staining. The sections were then incubated with avidin-peroxidase complexes in PBS (1:50) for 30 minutes and rinsed with PBS (3×5 minutes). Peroxidase activity was visualized with 3,3’-diaminobenzidine until the brown product was clearly visible. The sections were then counterstained with methyl green. The positive apoptotic cells were the cells with brown nucleus.\textsuperscript{20}

**Statistical analysis**

All data are expressed as the mean ±S.E.M. The differences in all parameters were analyzed by a one-way analysis of variance (ANOVA) followed by a Dunnett’s Multiple Comparison Test using sigma plot.\textsuperscript{11} A change was considered statistically significant if \( P<0.05. \)

**Results**

**Effect of aliskiren on body weight and blood glucose**

Before the treatment, there were no significant differences of baseline body weight of the rats (Table 1). In the aliskiren (10 mg/kg)-treated rats, a significant increase in body weight was noted (224.2±4.1g, \( P<0.001 \)), as compared with diabetic groups (198.3±3.1g) after eight weeks of study (Table 1).

Before treatment, there was significantly higher FPG (\( p < 0.001 \)) in all groups when compared with normal (Table 1). After eight weeks, groups treated with aliskiren showed a significant reduction of blood glucose versus the diabetic group (\( p < 0.01 \)) (Table 1).

**Effect of aliskiren on liver GLUT2 and muscle GLUT4 expression**

In diabetic rats, GLUT2 expression in liver and GLUT4 expression in soleus muscle was reduced; whereas aliskiren-treated rats showed improvement in both GLUT2 and GLUT4 expressions (Figure 1).

<table>
<thead>
<tr>
<th>Table 1. Effect of aliskiren on body weight and blood glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
</tr>
<tr>
<td>Before treatment</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Diabetic</td>
</tr>
<tr>
<td>Aliskiren (10 mg/kg)</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group. \(* * * * p < 0.001 vs. normal group, \( p < 0.05; \quad * p < 0.01; \quad ** p < 0.001 vs. diabetic group.\)
Biochemical markers

*Plasma insulin.* Aliskiren at the dose 10 mg/kg significantly increased plasma insulin \( p<0.01 \) levels in the treated diabetic rats as compared with the diabetic groups after eight weeks (Table 2).

Total proteins and albumin in plasma

Total proteins levels were found to increase significantly in plasma of the aliskiren-treated diabetic rats \( p<0.001 \), whereas the increase in plasma level of albumin was not statistically significant (Table 2).

Hemodynamic parameters

Aliskiren at the depressor dose of 10 mg/kg reduced the systolic blood pressure by 13.4% (Figure 2). The relationship between blood pressure and urinary protein excretion during treatment with aliskiren was further examined by correlation analysis. There was no correlation between blood pressure and serum protein in rats treated with a depressor-dose of aliskiren \( r = -0.194 \). In contrast, serum

Table 2. Effect of aliskiren administration on various biochemical and renal parameters

<table>
<thead>
<tr>
<th>Name of parameter</th>
<th>Normal</th>
<th>Diabetic</th>
<th>Aliskiren treated (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>17.83±3.2***</td>
<td>3.265±0.36</td>
<td>12.74±1.77**</td>
</tr>
<tr>
<td>Total proteins (mg/ml)</td>
<td>2.144±0.076***</td>
<td>1.368±0.17</td>
<td>2.029±0.1****</td>
</tr>
<tr>
<td>Serum albumin (ng/ml)</td>
<td>1.26±0.26*</td>
<td>0.1422±0.0151</td>
<td>0.8175±0.509</td>
</tr>
<tr>
<td><strong>GFR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.211±0.21****</td>
<td>3.589±0.18</td>
<td>1.753±0.27**</td>
</tr>
<tr>
<td>Serum cystatin c (ng/ml)</td>
<td>0.2298±0.026***</td>
<td>0.644±0.033</td>
<td>0.4628±0.058*</td>
</tr>
<tr>
<td>Beta-2 microglobulin (µg/ml)</td>
<td>0.4419±0.048**</td>
<td>1.362±0.12</td>
<td>0.6709±0.18*</td>
</tr>
<tr>
<td><strong>Inflammatory markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.0176±0.00545**</td>
<td>0.427±0.2198</td>
<td>0.08701±0.020*</td>
</tr>
<tr>
<td>TGF-β (pg/ml)</td>
<td>3.361±0.98****</td>
<td>11.3±1.348</td>
<td>6.673±0.44*</td>
</tr>
<tr>
<td>Nitric oxide (nmol/µl)</td>
<td>0.0176±0.00545**</td>
<td>0.427±0.2198</td>
<td>0.08701±0.020*</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>48.5±3.05****</td>
<td>74.08±5.34</td>
<td>64.88±1.16</td>
</tr>
<tr>
<td><strong>Molecular markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>3.393±0.21***</td>
<td>0.4±0.089</td>
<td>1.343±0.58</td>
</tr>
<tr>
<td>Erythropoietin (mIU/ml)</td>
<td>0.6775±0.026****</td>
<td>0.1283±0.064</td>
<td>0.4641±0.112*</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group. *p < 0.05; **p < 0.01; ***p < 0.001 vs. diabetic group. GFR: glomerular filtration rate; TNF-α: tumor necrosis factor alpha; TGF-β: transforming growth factor beta; VEGF: vascular endothelial growth factor.
albumin levels were strongly correlated with blood pressure in rats that were treated with depressor-dose aliskiren \((r = 0.92)\) (data not published).

**Glomerular filtration rate, renal proteins and inflammatory markers**

**Serum creatinine.** Diabetic control rats had elevated serum creatinine (normal: 1.79±0.21 mg/dl; diabetic: 3.59±0.27 mg/dl), which was significantly reduced with aliskiren \((P<0.01)\) after eight weeks of chronic dosing (Table 2).

**Serum cystatin c**

Serum cystatin c has been increased in the diabetic rats. Treatment of the STZ-induced diabetic nephropathy rats with aliskiren significantly decreased the serum cystatin c levels \((P<0.05)\) (Table 2).

**Beta-2 microglobulin**

The treatment with aliskiren caused a significant decrease in the serum beta-2 microglobulin concentrations \((p<0.01)\) in comparison with the diabetic control group (Table 2). The area under the receiver operating characteristic (ROC) curve for cystatin c was observed as 0.98 and that of creatinine was 0.67, indicating better diagnostic efficiency values for cystatin c than creatinine (Figure 3).

**TNF-alpha**

This can be further supported by the significant difference in the mean values of TNF-α observed in plasma between the normal control and diabetic group \((P<0.01)\) (Table 2). After the eight-week treatment with 10 mg/kg dose of aliskiren, the levels of TNF-α significantly decreased when compared with the levels in the diabetic rats \((P<0.01)\) (Table 2).

**NO**

NO levels were significantly increased in diabetic groups \((p<0.01)\). On treatment with aliskiren, NO levels were found to decrease in comparison with the diabetic group \((p<0.05)\) (Table 2).

**Growth factor**

**Transforming Growth Factor-β1 (TGF-β1)**. TGF-β1, a key participant in the development of kidney sclerosis, significantly differs in the diabetic and normal control groups \((P<0.001)\) (Table 2). The concentration of TGF-β1 was decreased significantly \((P<0.05)\) when compared with the diabetic group after eight weeks of treatment with aliskiren (Table 2).

**VEGF**

VEGF is a critical component in the tissue growth and organ repair processes of angiogenesis and vasculogenesis. VEGF was significantly lower in the control groups than in the diabetic group \((P<0.001)\). The aliskiren treatment showed lowering of VEGF levels in diabetic rats but data was not found to be statistically significant (Table 2).

**Molecular markers**

**Adiponectin.** Adiponectin was measured as a surrogate marker for inflammation and was significantly lower in the diabetic group than in the normal control group \((P<0.01)\). On treatment with aliskiren, adiponectin levels were increased but the augmentation was not found to be statistically significant (Table 2).
Erythropoietin

The erythropoietin concentrations were found to increase significantly \((P<0.05)\) after treatment with aliskiren for eight weeks when compared with the diabetic group (Table 2).

Histopathology

In control rats, the kidney cortex appeared normal with only some glomeruli displaying thickened glomerular basement membranes (GBM) (Figure 4). In contrast, in diabetic control rats most glomeruli exhibited thickened GBM, capillary occlusion and mesangial expansion. In addition, many cortical tubules were vacuolated (Figure 4). In diabetic rats treated with aliskiren, glomerular pathology was improved (Figure 4).

Conclusion

Diabetes was associated with an increase in GSI compared to diabetic control rats. GSI was lower in diabetic rats treated with aliskiren (Figure 4).

Quantitation of matrix deposition

In the tubule-interstitium of kidney cortex or medulla, increased collagen and inflammatory cells were observed in diabetic rats compared to normal rats. Compared to diabetic control rats, interstitial fibrosis was lower in diabetic rats treated with aliskiren monotherapy (Figure 4).

DNA fragmentation

There was an evident increase in the DNA fragmentation in the diabetic kidney compared to the normal kidney.
(Figure 4), whereas the kidney treated with aliskiren showed a clear decrease in the extent of DNA fragmentation (Figure 4).

**Discussion**

The direct inhibition of renin is a logical target for pharmacologic suppression of the RAAS because renin-mediated cleavage of angiotensinogen to form angiotensin I is a rate-limiting first step in the RAAS pathway. The neonatal-STZ Wistar model is a well characterized model of type 2 diabetes. Neonatal-STZ rats develop persistent diabetes rapidly after six weeks of age and show diabetes-like symptoms such as lack of insulin release in response to glucose, glucose intolerance, raised glycosylated hemoglobin and depletion of pancreatic insulin store.\(^{21-24}\) Further, when rendered diabetic with STZ, they develop renal damage considered analogous to that seen in human diabetic nephropathy.\(^ {25}\) At the same time, it has been said that diabetes induced by STZ injection significantly downregulates the protein expression of ACE-2 and Ang-(1–7) mas R, which eventually causes renal hypertrophy, tissue injury and apoptosis via the upregulation of phosphorylation of ERK1/2, p38 MAPK and JNK protein expression.\(^ {26}\)

The blood pressure-lowering efficacy of aliskiren in hypertensive patients is also well known and authenticated by various clinical trials.\(^ {8,9,27}\) Pilz et al. also demonstrated a protective effect of aliskiren for both heart and kidney injuries in double transgenic rats for human renin and angiotensinogen genes.\(^ {10}\) However, in a recent Aliskiren in the Evaluation of Proteinuria in Diabetes (AVOID) clinical study, aliskirenin combined with losartan had a significant anti-proteinuric effect that was independent of its blood pressure-lowering effect in patients with hypertension and diabetic nephropathy.\(^ {28,29}\) Kelly et al. illustrated that aliskiren had a similar efficacy to the angiotensin-converting enzyme (ACE) inhibitor in reducing urinary albumin and glomerulosclerosis in diabetic transgenic (mRen-2)27 rats.\(^ {11}\) Together, these results suggest a beneficial effect of renin inhibition in hypertensive and diabetic renal disease.

In the present study, we demonstrated that aliskiren treatment significantly improved insulin resistance and renal variables in STZ-induced diabetes without much effect on blood pressure. Aliskiren treatment also showed organ-protective effects such as improvement of functional and structural changes in the kidney. We also provided evidence that aliskiren could inhibit renin-mediated activation of profibrotic and angiogenic cytokine markers through an angiotensin-dependent mechanism.

The dosage of aliskiren used in the present investigation was selected on the basis of previous studies.\(^ {11}\)

In pursuit of that, the diabetic rats in the present study experienced approximately an 81% decrease in plasma/serum pancreatic insulin content, which was significantly improved with aliskiren treatment. At the same time, aliskiren treatment significantly reduced blood glucose and increased body weight as compared to diabetic rats.

The glucotransporter isoforms GLUT2 and GLUT4 play central roles in the complex pathways mediating whole body glucose disposal, in which dysregulation of their controlling mechanism can result in the pathophysiologic states associated with diabetes.\(^ {30}\) Aliskiren-treated diabetic rats showed improved liver and muscle glucotransporter expression levels qualitatively. These results qualify the improved insulin sensitivity of aliskiren. The findings are in accordance with a recent study that demonstrates that renin inhibition attenuates insulin resistance and improves systemic insulin sensitivity in transgenic Ren2 rats that over-express renin.\(^ {30,31}\) Thus, a possible link between renin activation and insulin resistance is suggested.

Furthermore, proteinuria, insulin resistance and formation of reactive oxygen species (ROS) are also associated with loss of adiponectin level in diabetic rats. STZ-induced diabetic rats showed almost an 87% decrease in adiponectin levels as compared with normal rats, which was ameliorated on treatment with aliskiren. Adiponectin can improve both glucose metabolism and insulin resistance via the AMP-activated protein kinase (AMPK) signaling pathway, both in liver and muscle, thereby lowering the blood glucose concentrations in vivo.\(^ {32,33}\) Studies have shown that phosphorylation of AMPK-α (thr172) is decreased in diabetic rats. The decreased plasma adiponectin levels may partly explain the decreased phosphorylation of AMPK-α (Thr172) in diabetic rats. Phosphorylation of AMPK increases glucose transport by stimulating the translocation of GLUT4.\(^ {26}\) The improvement of adiponectin levels in the diabetic treated rats can further be confirmed by the significant increase in body weight of the animals on aliskiren treatment, though the increase in adiponectin levels was not significant. Hence, we propose from our findings that RAAS blockade increased adiponectin concentrations with a consequent improvement in insulin sensitivity and secretion.\(^ {34}\) This was in accordance with the clinical study to assess plasma adiponectin as an independent predictor of type 2 diabetes especially in Asian Indians.\(^ {35}\)

Indeed, adiponectin has also been shown to accumulate in injured vessel walls and dose-dependently inhibit TNF-α-induced cell adhesion in human aortic endothelial cells.\(^ {36}\) Furthermore, TNF-alpha mRNA and protein levels were found to be increased in renal tissue from diabetic rats, which has been directly related to principal alterations of diabetic nephropathy as well as in cellular apoptosis and necrosis.\(^ {37}\) The present study confirms the significant reduction in levels of TNF-alpha in serum after eight weeks of chronic dosing with aliskiren when compared with the diabetic control group, thereby inhibiting apoptosis. Moreover, chronic hyperadiponectinemia had been shown to alleviate the progression of proteinuria in early-stage diabetic nephropathy. The mechanism whereby adiponectin decreases proteinuria has been shown to involve
an increase in nephrin expression, and demonstrates an improvement of the endothelial dysfunction because of decreases in ET-1 and PAI-1 in the renal cortex.58

Hence, we also tried to explore the changes in renal function on treatment with the depressor dose of aliskiren. Increased urinary albumin excretion (UAE) is the hallmark of diabetic nephropathy. Studies indicate that albuminuria is a major renal and cardiovascular risk factor in patients with diabetes, and that the degree of albuminuria reduction under treatment is critical in terms of long-term clinical outcome.59–41 Interventions that have attenuated the progression of diabetic nephropathy have been associated with a reduction in urinary protein excretion,42 and thus renoprotective therapy should aim to achieve the maximal anti-albuminuric effect.43 However, a recent study proposed that in 20 diabetic patients with macroalbuminuria, large quantities of protein fragments, not routinely measured clinically, were present.44 Also, lower serum albumin concentration imposes a greater increase in relative risk of mortality among populations as serum values of albumin are not readily affected by minor variations by the body’s natural mechanism of maintaining homeostasis in albumin metabolism.45 Albumin, being a major source of sulphhydril groups,46 may be important in the activation of a systemic inflammatory response. Hence, we chose serum/plasma albumin as an important marker for identification of progression of renal damage. In our study, treatment of STZ-induced diabetic rats with aliskiren prevented the development of albuminuria that was seen in vehicle-treated diabetic controls, as the plasma albumin levels were found to increase after eight weeks of treatment with aliskiren, despite negligible differences in blood pressure in treated diabetic rats. Further, decrease in plasma proteins may also elicit pro-inflammatory and pro-fibrotic effects that directly contribute to chronic tubulo-interstitial damage.47 Consequently, an increase in protein and albumin levels was achieved with obvious drug effect on glucose metabolism and insulin levels. However, the increase in either was at least partially independent of the decrease in blood pressure as the reduction is desirable in diabetic nephropathy, a microvascular complication, where patients are normally observed to be hypertensive. This BP independent effect was verified by examining the correlation between individual values of BP and serum total protein. The variance of serum albumin in this group is due to the effects of aliskiren on systolic BP, suggesting that intrarenal effects may also play an important role along with systemic blood pressure in manipulating the renal variables with this drug. This finding is consistent with those of earlier published preclinical and clinical studies.48,49 Moreover, it is still not confirmed whether these beneficial actions of aliskiren are due to its antihypertensive effect or renin inhibition. Nonetheless, it has also been reported that renin inhibition with aliskiren attenuated renal vascular resistance and significantly reduced BP in diabetic patients, which was observed in our study also. As monotherapy, aliskiren has been shown to be equally effective as an ACE inhibitor or ARB in lowering BP, but not more effective.50–52 Several explanations can be proposed,53 but the simplest is that the renin-angiotensin system is only one of the many regulatory pathways contributing to hypertension. The modest BP reduction with aliskiren monotherapy most likely reflects compensation. Regardless of the precise explanation, aliskiren does not eliminate the need for multi-drug regimens to control most cases of hypertension, nor is it expected to be a magic bullet for resistant hypertension and renal complications in diabetic patients.

Additionally, the aliskiren treatment was found to decrease the NO levels in the study undertaken, which could be because of an increase in the renal expression of the p47phox component of NAD (P) H oxidase and eNOS, thereby decreasing the indices of systemic and renal oxidative/nitrosative stress as proposed by Sonta et al. in 2005.54 Besides, kidney also has a finite capacity to regulate intraglomerular pressure so that some transmission of systemic pressure to the glomerular tuft still occurs. These effects are magnified by the local synthesis of angiotensin II, which by preferentially constricting the effluent arteriole, raises intra-glomerular pressure. Further, it induces the expression of fibrotic growth factors, namely TGF-β1 and VEGF via MAPK p42/p44, thereby upregulating molecules like fibronectin, collagen-1 and plasminogen-activator inhibitor-1 (PAI-1),55–57 leading to glomerulosclerosis.58–60 In the present study, aliskiren reduced glomerulosclerosis significantly, which is further supported by significant reduction of TGF-β1 and VEGF. VEGF in glomeruli has been associated with glomerular hypertrophy and increased production of alpha3 (IV) collagen, which is an integral component of the GBM, thereby leading to sclerosis. The findings were consistent with a two-fold increase of glomerular VEGF mRNA in transgenic (mRen-2)27 rats.61 Aliskiren treatment in the present investigation also attenuated tubulointerstitial fibrosis, another important predictor of renal dysfunction,62 which has been explained by the prevention of desmin expression, which preserves podocytes and protects against interstitial fibrosis.63

In addition, anemia has been associated with an accelerated decline in renal function in some of the patient groups. Decreased tissue oxygen delivery caused by anemia stimulates the RAAS and contributes to renal vasoconstriction. These factors can further exacerbate proteinuria, which may worsen renal function. In patients with type 2 diabetes, anemia has been shown to be an independent risk factor for progression of renal disease.64 The improvement of erythropoietin levels with aliskiren treatment shows a wide range of effects produced by the aliskiren treatment in delaying the progression to renal complications.

Further, to substantiate the renal variables, measuring GFR is widely accepted as the best overall index of kidney function. In clinical practice, an approximation of GFR is
often obtained from plasma/urine creatinine concentration alone, albeit with limited accuracy. Pure and reliable urinary samples are very challenging to obtain from experimental animals, especially from small rodents. Moreover, serum creatinine is particularly insensitive for identifying chronic kidney disease in its early to middle stages and in certain patient groups (e.g. children, females, elderly). It is also considered relatively specific but not very sensitive since its levels significantly increase only when more than 50% of the GFR is reduced. Measurement of cystatin c or beta2-microglobulin (B2M) concentrations has been found to be advantageous over serum creatinine concentration for the detection of an impaired GFR. Cystatin c and B2M are low-molecular weight endogenous proteins freely filtered by the glomerulus and proximal tubule, respectively. It is already shown that serum cystatin c levels rise earlier and more rapidly than those of serum creatinine as GFR decreases, reflecting greater sensitivity and hence diagnostic accuracy (related to GFR measured as $^{51}$Cr-EDTA clearance) of cystatin c as a predictor of GFR in type 2 diabetic patients. Also, serum cystatin c level is correlated strongly with 24-hour creatinine clearance ($r = 0.832$, $p <0.001$). ROC plot analysis in our study confirms both of these findings of cystatin c being a sensitive diagnostic marker for renal impairment with an area under the curve (AUC; 95% confidence interval (CI)) of 0.98 being superior to that of serum creatinine (0.67, 95% CI). Further, beta-2 microglobulin, another endogenous filtration marker, by virtue of its retention due to renal function failure, is deposited in tissues and aggregated into fibrils, and hence become glycosylated. However, B2M is unstable in acid and degrades in infected alkaline urine during the time when the urine is held in the bladder; hence its absence cannot be relied on to exclude the presence of a tubular proteinuria. Therefore, we performed the analysis of the filtration markers in serum. Also their serum concentrations are less dependent on extra renal factors than serum creatinine. In nephrotic syndrome, the increased excretion of low molecular weight plasma protein is probably explained by accompanying tubular malfunction, but in renal failure the overwhelming reabsorptive capacity in the less-affected nephrons is the major factor. Virtually, studies have shown that patients excreting increased quantities of low molecular weight proteins were also excreting increased amounts of albumin, so an increased albumin excretion does not necessarily indicate glomerular disease. In the present study conducted, it was clear that monotherapy with aliskiren improved the creatinine clearance, which is evident from the significant decrease in serum creatinine values. At the same time, serum levels of cystatin c and BMG were significantly decreased when compared with diabetic rats after chronic administration of aliskiren. These findings were consistent with the earlier study done by Byung et al. in 2007. Further, even mild renal impairment is strongly associated with increased mortality in patients; the ROC analysis validates the sensitivity and specificity of cystatin c measurement to assess the substantially reduced GFR effectively, thereby reducing the progression of morbidity and mortality by aliskiren treatment and hence justifying its efficacy in renal complication of diabetes over creatinine-based measures.

Conclusion

The present study authenticates the use of direct renin inhibition (DRI) as a renoprotective agent, in addition to its utility as an antihypertensive. The ability to provide a more complete inhibition of RAAS by overcoming the expected limitations of angiotensin II receptor blockers and ACE inhibitors, and thereby preventing possible renal-damaging direct effects of renin including apoptosis of the kidney, make DRIs appealing to nephrologists. Nevertheless, the study cannot rule out the use of a combination of drugs to provide an effective blockade of the renin angiotensin system, thereby providing more efficient renoprotection by inhibiting angiotensin-dependent and independent pathways with slight changes in BP. The elevation of prorenin as a compensatory mechanism in patients with diabetic nephropathy further needs special attention and could make it unsafe in the long run. However, better glucose translocation in liver and muscle along with renal variables testifies to the beneficial use of aliskiren in diabetic complications.

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Conflict of interest statement

None declared.

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