Treatment with NZ-419 (5-Hydroxy-1-methylimidazole-2,4-dione), a Novel Intrinsic Antioxidant, against the Progression of Chronic Kidney Disease at Stages 3 and 4 in Rats

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For rats, glomerular filtration rate (GFR) and its relative GFR (ratio to normal GFR) were estimated in order to classify their chronic kidney disease (CKD) into 5 stages like those in humans. The adenine-loaded rats, which were used to show the intrinsic antioxidant and creatinine (Cr) metabolite, NZ-419 (5-hydroxy-1-methylimidazole-2,4-dione), when taken orally, prevented the progression of chronic renal failure (CRF), were used as a model to reach the severest stage 5. In this report, we show that, by using both a tubular lesion and a glomerular lesion models (adenine-loaded and 5/6 nephrectomized rats, respectively), peroral NZ-419 might be a common tool to prevent the progression of CRF at CKD stages 3 and 4 under the condition that most rats in the control group still remained at stage 4 (0.15< GFR/GFR0< 0.29) at the last. At the minimum effective dose (MED: 100 mg/kg/d) of NZ-419 in adenine-loaded rats, serum Cr and all oxidative stress markers were ameliorated. Two doses (80, 160 mg/kg/d), at around the MED, used for 5/6 nephrectomized rats with a similar CRF severity, gave significant inhibitory effects against the increases in blood urea nitrogen, decreases in renal blood flow and renal plasma flow, and nephrotic syndrome. Oxidative stress markers, the urinary methylglycine-dimer and serum albumin level, were significantly ameliorated.

Key words NZ-419; 5-hydroxy-1-methylhydantoin; creatinine metabolite; intrinsic anti-oxidant; chronic renal failure; chronic kidney disease

Once the irreversible process of chronic renal failure (CRF) is established, from whatever causative diseases, a progressive course follows ultimately leading to the cessation of renal function and end-stage renal disease (ESRD). Recently the concept of chronic kidney diseases (CKD) and its classification into five stages has become popular among medical practitioners since 2002 (Table 1).1) Treatments of patients have been indicated to be different between stage 1 or 2 and 3, 4 or 5; at the former stage, 1 or 2, kidney damage is important but decrease in glomerular filtration rate (GFR) is still not practically crucial, but at the latter stage, 3, 4 or 5, decrease in GFR is serious and CRF cannot be ignored,1) as hydroxyl radicals increase markedly.2) Effective treatments of CRF patients or CKD patients at stages 3, 4 and 5 before ESRD have been widely recognized as essential and urgent targets.3) In our previous paper,3) we reported for the first time that an intrinsic antioxidant compound could prevent the initiation and/or progression of CRF: the compound investigated was NZ-419 (5-hydroxy-1-methylimidazole-2,4-dione, 5-hydroxy-1-methylhydantoin: HMH, CAS: 84210-26-4),4–9) a creatinine (Cr) metabolite that prevented the initiation and/or progression of adenine-induced CRF. We chose Cr or CKD at stages equivalent to human stages 3, 4 and 5, and hydroxyl radicals are over-produced,2) as one of the targets aims.3,5,11,12) because NZ-419 had been shown to be a hydroxyl radical scavenger.11,12) Meanwhile, since there is a hypothesis that oxidative stress might be a common key factor in the progression of CRF induced by several different causes, antioxidants might inhibit the progression of CRF regardless of its causes.3) In order to assess whether NZ-419 could be used as a common treatment for CRF at CKD stages equivalent to human ones, 3, 4 and 5, its effect on CRF with other causes must be also investigated. Since adenine-induced CRF rats5,13) have been shown to be a tubular lesion model, (as a glomerular lesion model) an additional model of 5/6 nephrectomized rats15) was used together with the adenine-loaded rats in this study. Because NZ-419 might not be an agent to delete uremic toxins and CKD stage 5 might be too late to be treated with NZ-419, we thought stages 3 and 4 might be the best stages for treatment timing for CKD. And so we chose the conditions which might not lead most rats to stage 5. Firstly, we investigated the minimum effective dose (MED) of NZ-419 in adenine-loaded rats, and then its efficacy in nephrectomized rats at doses around the MED. The hyperfiltration theory states that, as nephrons are gradually lost in renal failure, the remaining nephrons become hypertrophied to compensate for the loss of renal function, and hyperfiltration in these remaining nephrons leads to further nephron losses.16) These nephron losses induce a reduction of renal function such as via progressive proteinuria and a declining GFR. Nephron loss is caused by glomerulosclerosis, tubulointerstitial fibrosis, the loss of glomerular cells, and tubular atrophy.17)

Now some effective treatments including dietary control, drug therapy with a spherical carbonaceous absorbent, and anti-hypertensive drugs including ACEIs (angiotensin-converting enzyme inhibitors) and ARBs (angiotensin receptor blockades) were available to inhibit the progression of CKD and CRF, but these were still not effective enough. Therefore, the development of new drugs that prevent the progression of CKD and CRF with a new mechanism is still an urgent requirement.

Although the mechanism leading to the progression of CKD and CRF has not yet been fully elucidated, other common mechanisms have been considered. Among them, oxidative stress-related mechanisms have been widely studied, as we cited in our previous paper.3) Antioxidants have shown ef-
fficacy, but not many are safe to use.\(^{1,19}\) As candidates, we have already reported that tannins, antioxidants in green tea and crude drug etc., showed inhibitory effects on the progression of CRF in adenine-loaded and nephrectomized rats.\(^{20—22}\) In our previous paper,\(^{3}\) we reported that NZ-419 could be one such safe candidate because crucial side effects were not observed at doses less than 1000 mg/kg/d.

**MATERIALS AND METHODS**

**Estimation of GFR for Rats** Clinically, GFR and Cr clearance (CCr) have been successively estimated from the corresponding serum Cr (sCr) value,\(^{1}\) if we consider the coefficient for gender, race, age, and body surface area or weight. Since, in our reported papers,\(^{23—25}\) only male Wistar rats with a similar age were used, a simple correlation equation between the ratio CCR/CCR\(_0\) (CCR\(_0\): CCR of normal rats) and sCr was obtained using the reported corresponding mean values of various groups with different renal functions.\(^{23—25}\) We hypothesized that GFR/GFR\(_0\) as well as GFR, GFR\(_0\) (GFR of normal rats) is nearly equal to corresponding CCR/CCR\(_0\), CCR, and CCR\(_0\), respectively.

We have already measured six kinds of values at the same time: values of GFR, renal plasma flow (RPF) and renal blood flow (RBF) for CRF rats plus those of normal rats (GFR\(_0\), RPF\(_0\) and RBF\(_0\), respectively).\(^{23,24}\) We also obtained correlations between relative renal functions such as GFR/GFR\(_0\) and RPF/RPF\(_0\) or RBF/RBF\(_0\) using the reported mean values of various groups with different renal functions in our paper.

After GFR/GFR\(_0\) was estimated by three methods mentioned above via sCr, RPF/RPF\(_0\) or RBF/RBF\(_0\), since rat GFR\(_0\) has been reported to be ca. 0.55 ml/min/kg,\(^{23}\) estimated GFR (eGFR) could be calculated from GFR/GFR\(_0\).

**Comparison of Renal Function-Related Parameters between Animals and Clinical Cases** Clinically, CKD patients are now classified into five stages using the GFR (Table 1).\(^{1,11}\) Stages of CRF patients are shown to be 3, 4, or 5: eGFR of these are 30—59, 15—29, or <15 ml/min/1.73 cm\(^2\), respectively. Since the clinical eGFR\(_0\) has been shown to be ca. 100 ml/min/1.73 cm\(^2\),\(^{26}\) the relative renal function, eGFR/ eGFR\(_0\), of patients in stages 3, 4, and 5 would be 0.30—0.59, 0.15—0.29, or <0.15, respectively. So, we divided rats with similar stages to humans using the relative renal function, GFR/GFR\(_0\) (Table 1).

**Animals and Chemicals** The Guidelines for Animal Experimentation, approved by the University of Toyama, were followed in these experiments. Male Wistar rats (ca. 200—210 g), obtained from Japan SLC, Inc. (Hamamatsu, Japan), were used. They were maintained at a constant humidity (ca. 60%) and temperature (ca. 23°C) with a light-dark cycle of 12 h. NZ-419 was prepared in the Institute of Bio-Active Science (IBAS: Nippon-Zoki, Hyogo, Japan). Other reagents were of an analytically pure or HPLC grade.

**Experiment Using Adenine-Loaded CRF Rats. (A) Experimental Design** Rats were allowed an adaptation period of several days, during which time they were fed a commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan). They were then fed by adopting a pair-feeding schedule on an 18% casein diet containing 0.75% adenine, which experimentally induced renal failure in the animals.\(^{1,13}\) During the adenine-feeding period, an aqueous solution of NZ-419 (25, 50, 100 mg/kg) was administered orally for 20 d in drinking water, while control rats received tap water.\(^{3}\)

**B) Determination of Serum Components** Urea nitrogen, total protein, albumin, inorganic phosphate, and calcium were determined using commercial reagents (BUN Kainos, from Kainos Laboratories, Inc., Tokyo, Japan; A/G B-Test Wako, Phosphor B-Test Wako, and Calcium C-Test Wako, from Wako Pure Chemical Industries, Ltd., Osaka, Japan). Concentrations of Cr, methylguanidine (MG), and guanidino-succinic acid (GSA) were analyzed with a guanidino-com pound analyzing system (Japan Spectroscopic Co., Tokyo, Japan) by HPLC using the fluorogenic reagent 9,10-phenanthroquinone.

**C) Determination of MED** Among the MED for parameters measured, the MED of sCr was chosen to be the MED for this adenine-loaded model.

**Experiment Using 5/6 Nephrectomized Rats. (A) Experimental Design** Rats weighing about 200 g were resectioned leaving of 2/3 of the left kidney and total excision of the right kidney with an inter-operative interval of 10 to 14 d. The blood urea nitrogen level of the rats was determined after their recovery from the operation, and they were divided into three groups to avoid any inter-group difference in the blood urea nitrogen level. The first group was given water, while the other two groups were administered orally with NZ-419 at 80 or 160 mg/kg body weight/d in drinking water for 90 consecutive days. We chose these two doses, 80 and 160 mg/kg/d, in order to sandwich the MED (100 mg/kg/d) for the adenine-loaded model. The dose was adjusted by regulating the concentration in relation to water consumption. To ensure that the food intake was constant among the three groups, the animals were raised on a pair-feeding schedule. Blood urea nitrogen and urinary protein excretion were determined every 10 or 20, and 30 d, respectively, during the administration period. From day 89 to 90, 24-h urine was collected. On the 90th day, the renal function was evaluated as

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Clinical GFR(^{a,c}) (ml/min/1.73 m(^2))</th>
<th>Relative GFR(\times)GFR(_0) (GFR/GFR(_0))</th>
<th>Rat GFR(^{b}) (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal or ↑ GFR</td>
<td>≥90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mild ↓ GFR</td>
<td>60—89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate ↓ GFR</td>
<td>30—59</td>
<td>0.30—0.59</td>
<td>0.16—0.31</td>
</tr>
<tr>
<td>4</td>
<td>Severe ↓ GFR</td>
<td>15—29</td>
<td>0.15—0.29</td>
<td>0.08—0.15</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure (&lt;15 or dialysis)</td>
<td></td>
<td>&lt;0.15</td>
<td>&lt;0.08</td>
</tr>
</tbody>
</table>

\(^{a}\) Clinical GFR\(_0\), GFR of normal subjects, has been reported to be about 100 ml/min/1.73 m\(^2\). \(^{b}\) Rat GFR\(_0\), GFR of normal rats, has been reported to be about ca. 0.55 ml/min/kg. \(^{c}\) The National Kidney Foundation K/DOQI Clinical Practice Guidelines on Chronic Kidney Disease: Am J Kidney Dis., 39 (2 Suppl. 1), S1—S266 (2002).
shown below, and blood was collected as shown above in an adenine-loaded case. The residual kidney was completely perfused with pre-cooled saline and removed.

**B** (B) Determination of Serum and Urine Components

Urea nitrogen, total protein, albumin, Cr, MG, and GSA were determined as described above. Urinary protein was determined by the method of Lowry et al., using bovine serum albumin as a standard.27)

**C** (C) Determination of Renal Function Parameters

RPF was measured by employing the renal clearance test using a single intravenous dose of sodium para-aminohippurate as an indicator.29) At 25 min after intravenous administration of the agent, the bladder was reflexly emptied by making each rat inhale ether for 3—5 s. The urine so voided was discarded. During the next 30 min, the urine was collected, and collection was terminated after the bladder had again been emptied reflexly by ether inhalation. Blood samples were taken from the conscious rats by heart puncture in the middle of the period used for the clearance test. para-Aminohippurate was determined by colorimetry. RBF was calculated on the basis of RPF and the hematocrit value (Ht) using the equation below. Ht was determined with an Ht measurement apparatus, model KH-120A (Kubota Co., Ltd., Tokyo, Japan): RBF = RPF/(1 − Ht) (ml/min).24)

**D** (D) Histopathological Examination

The other part of the renal tissues was fixed in Bouin’s solution, embedded in paraffin, and cut into thin sections. These sections were stained with hematoxylin–eosin, periodic acid-Schiff, or periodic acid methenamine silver stain, and observed by optical microscopy. Mesangial proliferation was rated as normal, slight, moderate, or severe. The proportion of sclerotic lesions in each glomerulus was rated as grade 0—4, using the method of Raij et al.,29) where grade 1 represents the involvement of up to 25% of the glomerulus and grade 4 represents sclerosis of 75—100% of the glomerulus. The glomerular sclerosis index was obtained by averaging the scores for all glomeruli from each rat. The severity of tubulo-interstitial lesions was assessed according to three degrees: normal, mild, or severe. Rats from which 50 or fewer glomeruli were obtained were excluded from analysis.

**Statistical Analysis** Data are expressed as means±S.E.M. The significance of differences between experimental groups was determined using Student’s t-test and Scheffe’s multiple comparison test. The level of significance was set at p<0.05.

**RESULTS**

**Estimation of GFR for Rats and Comparison of Renal Function-Related Parameters between Animals and Clinical Cases** As shown in Fig. 1, a simple correlation between Ccr/Ccr0 (x) and sCr (y) was as follows:

\[ y = 0.47x + 0.035 \quad (n=12 \text{ groups}: R^2=0.97) \quad (1) \]

If y equals 0.60, 0.30, or 0.15, x must be 0.74, 1.41, or 2.55, respectively. As shown in Table 1, rats at stages 3, 4, and 5 might be estimated roughly to have sCr values of 0.73—1.41, 1.40—2.55, and >2.55 mg/dl, respectively.

As shown in Fig. 2, RPF/RPF0 and RBF/RBF0 started decreasing at the stage 3.

![Fig. 1. Correlation between Ccr/Ccr0 and sCr](image1)

![Fig. 2. GFR/GFR0 and RPF/RPF0 or RBF/RBF0](image2)
Fig. 3, all values related to the renal function, except for the Ca level, in rats given with 100 mg/kg of NZ-419 were significantly better than the control levels. BUN and inorganic phosphate levels as well as MG and GSA levels showed that the dose of 50 mg/kg was also effective. Under this condition, 25 mg/kg of NZ-419 induced significantly better serum albumin level than the control level. These results suggested that the MED might be 50 or 100 mg/kg. Since sCr level is the most important one clinically evaluating a renal function, the MED was determined to be 100 mg/kg.

Effects of NZ-419 on 5/6 Nephrectomized Rats. (1) Blood Urea Nitrogen The BUN level in the nephrectomized control rats was maintained around the initial level of 48.8 mg/dl (normal level: 12.0 mg/dl) until day 30, and then increased to 63.8, 63.0, and 95.6 mg/dl at 50, 70, and 90 d, respectively, reflecting chronic progressive uremia, as shown in Fig. 4. The administration of NZ-419 at doses of 80 and 160 mg/kg of body weight/d significantly reduced the blood urea nitrogen level after 20 d.

(2) Urinary Protein The urinary protein level in the nephrectomized control rats increased from the initial level of 2.4 mg/d: 36.7, 98.2, and 87.2 mg/d at 30, 60, and 90 d, respectively, reflecting progressive CRF, as shown in Fig. 5A. The administration of NZ-419 at doses of 80 and 160 mg/kg of body weight/d significantly reduced the urinary protein level on any of the tested days.

(3) Total Protein, Albumin, and Total Cholesterol in Serum As nephrotic syndrome, not only an increase in urinary protein (as shown above) but also, in serum, a decrease in total protein and albumin (control level: 3.20±0.07 g/dl, n=10; reported normal level: 3.63±0.07 g/dl, n=6\(^12\)), and an increase in total cholesterol (control level: 152.9±14.9 mg/dl, n=10; reported normal level: 80.9±4.2 mg/dl, n=6\(^12\)) were observed at 90 d (Fig. 5B). NZ-419 significantly inhibited each abnormality in total protein and albumin in serum at both doses (80 and 160 mg/kg of body weight/d), and total cholesterol at the higher dose.

(4) Serum and Urinary Levels of Cr, MG, and GSA As shown in Fig. 6, the serum Cr and MG levels in the nephrectomized control rats increased to 2.23±0.17 mg/dl (n=10) and 2.44±0.95 µg/dl (n=10), respectively, at 90 d (normal rats: Cr level, 0.47 mg/dl; MG: not detectable).\(^14\) The relative renal function, GFR/GFR\(_0\) was estimated from this mean sCr value using equation 1, to be 0.18 (at stage 4). Although both mean values decreased with NZ-419 treatment, a significant reduction was not observed. The urinary level of Cr in the control rats decreased, and NZ-419 increased the level but not significantly. In a case of MG, a hydroxyl radical related marker, the urinary level of the control increased to be 46.0±1.1 µg/d (n=8) and NZ-419 inhibited the increase in a dose-dependent manner, and the decrease at a dose of 160 mg/kg to be 39.9±1.2 µg/d (n=9) was significantly. The serum and urinary levels of GSA in the control group increased (37.7±16.3 µg/dl (n=10) and 404.2±23.2 µg/dl (n=10), respectively), but were not much larger than those in adenine-loaded rats (serum level: 59.7±5.9 µg/dl (n=10). NZ-419 inhibited the increase but not significantly (Fig. 6).

(5) Renal Function Parameters, RPF and RBF The RPF and RBF values in the nephrectomized control rats de-
creased to 3.45 and 6.05 ml/min/kg, respectively (normal level: 20.8 and 36.9 ml/min/kg, respectively). Relative renal function ratio GFR/GFR₀ of the control rats was calculated to be 0.21 or 0.18, respectively, at stage 4, by using Eq. 2 and RPF/RPF₀, 0.17 (3.45/20.8) or Eq. 3 and RBF/RBF₀ value, 0.16 (6.05/36.9). Both doses of NZ-419 (80, 160 mg/kg) significantly inhibited the decrease: corresponding RPF values were 6.43 and 6.16 ml/min/kg, respectively; RBF values were 11.48 and 11.22 ml/min/kg, respectively (Fig. 7). Similarly, GFR/GFR₀ was calculated; for NZ-419 (80 mg/kg) rats: 0.28 or 0.26, respectively, by using Eq. 2 and RPF/RPF₀, 0.31 (6.43/20.8) or Eq. 3 and RBF/RBF₀ value, 0.31 (11.48/36.9), respectively; for NZ-419 (160 mg/kg) rats: 0.27 or 0.25, respectively, by using Eq. 2 and RPF/RPF₀, 0.30 (6.16/20.8) or Eq. 3 and RBF/RBF₀ 0.30 (11.22/36.9), respectively. Ht values of the control group and NZ-419 (80, 160 mg/kg) groups were 42.5±1.0% (n=8), 43.7±0.8% (n=7), and 45.2±0.7% (n=5), respectively.

(6) Histological Findings The glomerular sclerosis index increased and extra-capillary lesions occurred in the control rats, as previously reported. NZ-419 inhibited the histological changes, but not significantly under the conditions. As illustrated in Fig. 8, typical morphological changes, which are not seen in normal cases, were seen in the examined kidneys of the control group: glomerular changes, such as moderate mesangial proliferation, were observed as well as tubular expansion and interstitial fibrosis. In contrast, almost normal glomeruli and tubular interstitial figures were seen in the kidneys in the 160 mg NZ-419 group. The glomerular sclerosis index was reduced by 20% in both 80
and 160 mg NZ-419 groups, and the incidence of extracapillary lesions was inhibited by 38% at both doses. However, these effects under the conditions examined were not significant, as shown in Fig. 8.

DISCUSSION

The renal function, such as GFR, RPF, and RBF, of adenine-loaded rats has been reported to decrease to less than 10% of the normal level (GFR/GFR
\_0, RPF/RPF
\_0 or RBF/RBF
\_0 < 0.01) after ca. 24 d of adenine loading,\(^{24}\) although these abnormalities in RPF and RBF were estimated for the first time at stages equivalent to clinical CKD stages 3, 4, and 5 after the relative GFR (GFR/GFR
\_0) decreased under 0.60 (Fig. 2). We showed in the previous paper that there was an inhibitory effect of NZ-419 against the initiation and/or progression of CRF using the same 24 d loading conditions: MED seemed 250 mg/kg/d.\(^{3}\) In order to clarify the role in the beneficial effect of NZ-419,\(^{3}\) As explained above, determination of not only the MED (at 100 mg/kg/d) for adenine loaded rats but also the values of two other doses (at 80 and 160 mg/kg/d) for 5/6 nephrectomized rats gave the predicted results.

In 5/6 nephrectomized rats, the blood urea nitrogen level was maintained around the initial level until 30 d, and increased until 90 d, reflecting chronic progressive uremia. The administration of NZ-419 significantly reduced the BUN level. The urinary protein level in the nephrectomized control rats increased from the initial to high levels at 60 and 90 d. The administration of NZ-419 significantly reduced the increase in urinary protein. On the other hand, the renal function of nephrectomized rats decreased as much as in the adenine-loaded rats in this report. The relative renal function GFR/GFR
\_0 was estimated from the sCr, RPF/RPF
\_0, or RBF/RBF
\_0 values to be 0.18, 0.21 or 0.18, respectively, which indicated that most rats in the control group still remained in stage 4. As mentioned above, decrease in GFR/GFR
\_0 was indicated to be inhibited with NZ-419. Although we could not confirm the significant efficacy of NZ-419 on sCr, we can conclude from the renal function-related data such as RPF, RBF, and urinary protein, that NZ-419 prevented the progression of CKD. The CKD stages remain at 3 and 4, with around 100 mg/kg/d of dose which indicated prevention of the progression of CRF in rat models.

In a tubular lesion model, where the main production site of MG is in the proximal tubule, over-production of MG is marked. Furthermore, in the adenine-loaded model, uniform CRF rats could be easily obtained and inhibition of MG production by NZ-419 could be shown clearly. In contrast, in a glomerular lesion model, after protein is leaked, reabsorption and catabolism in tubules follows, and then generation of active oxygen species in the tubules becomes apparent at stages 3, 4 and 5; and so increase in MG production is less than in a tubular lesion model. Furthermore, without a selection of rats, uniform nephrectomized rats are difficult to be obtained. Therefore, effects of test compounds on MG are likely to be more difficult to be observed.

In comparison with adenine loaded rats having the same GFR, since less hydroxyl radical level in sera has been indicated in the 5/6 nephrectomized rats,\(^{24}\) lower serum level of MG with a larger standard error was observed. Therefore we could not observe significant difference, although decrease in MG was in a dose-dependent manner. In contrast, the urinary...
REFERENCES


