A Novel Sartan Derivative With Very Low Angiotensin II Type 1 Receptor Affinity Protects the Kidney in Type 2 Diabetic Rats

Yuko Izuhara, Toshio Sada, Hiroaki Yanagisawa, Hiroyuki Koike, Shuichi Ohtomo, Takashi Dan, Sadayoshi Ito, Masaomi Nangaku, Charles van Ypersele de Strihou, Toshio Miyata

Background—Antihypertensive angiotensin II receptor blockers (ARBs) protect the kidney, at least in part, independently of blood pressure lowering. Still, the extent to which blood pressure lowering is related to renoprotection remains unclear.

Methods and Results—139 newly synthesized ARB-derivatives were assayed for inhibition of advanced glycation (AGEs). The 9 most powerful compounds were then tested for transition metal chelation, angiotensin II type 1 receptor (AT1R) affinity, and pharmacokinetic parameters. R-147176 was eventually selected as it strongly inhibits advanced glycation but is 6700 times less effective than olmesartan in AT1R binding. It is orally bioavailable and toxicologically safe.

Despite a minimal blood pressure lowering effect, it provides significant renoprotection in 3 experimental rat models with renal injury, ie, obese, hypertensive, type 2 diabetic rats (SHR/NDmcr-cp), normotensive type 2 diabetic rats (Zucker diabetic fatty), and remnant kidney rats.

Conclusion—R-147176 retains renal protective properties despite a minimal blood pressure-lowering effect. Clearly, the renal benefits of ARBs do not necessarily depend on blood pressure lowering and AT1R affinity, but rather on the inhibition of AGEs and oxidative stress inherent to their chemical structure. R-147176 opens new avenues in the treatment of cardiovascular and kidney diseases. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

Key Words: blood pressure ■ renoprotection ■ advanced glycation end products ■ oxidative stress ■ type 2 diabetes

Angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) lower both blood pressure and proteinuria in diabetic patients.1–4 It is as yet unclear to what extent decreased proteinuria relates to blood pressure lowering. Indirect evidence suggests that it is, at least partially, independent of blood pressure control.5–8 Interestingly, we recently confirmed that, in experimental animals, higher doses of these drugs further lower proteinuria despite the absence of further blood pressure control.9

We10–12 and others13 have provided experimental evidence that ARBs also decrease renal oxidative stress and advanced glycation, a fact that might help understand the link between ARBs and improved proteinuria.

The present study has been undertaken to provide new insights into the interplay among blood pressure lowering, the inhibition of oxidative stress and advanced glycation shared, and resulting renoprotection. We designed an ARB-derivative, R-147176, with a marked inhibitory effect on oxidative stress and advanced glycation but a minimal affinity for the angiotensin II type 1 receptor (AT1R) and thus little antihypertensive effect. We tested its renoprotective benefits in 3 different experimental rat models, 2 with type 2 diabetic nephropathy, ie, the spontaneously hypertensive/NIH-corpulent (SHR/NDmcr-cp) and the Zucker diabetic fatty (ZDF) rats, and 1 with a remnant kidney.

Materials and Methods

See the supplemental data section at http://atvb.ahajournals.org for detailed Methods.

Strategy to Develop a New Compound

New ARB-derivatives were synthesized in R&D Division of Daiichi-Sankyo Co Ltd (Tokyo, Japan). They were screened in vitro for AGE inhibition by a previously described method.10–12 The most promising compounds were eventually tested for in vitro AT1R binding.

Inhibition of Pentosidine, an AGE

Fresh heparinized plasma samples were obtained after informed consent from hemodialysis patients before the dialysis session. Pooled plasma was incubated with the tested reagents (final concentration of 0.8, 2.0, and 5.0 mmol/L) for 1 week under air at 37°C. The content of pentosidine14 was analyzed on a C18 reverse phase...
high-performance liquid chromatography (HPLC) as previously described. Synthetic pentosidine was used as a standard.

Transition Metal Chelating Assay
The chelating activity of the tested compounds for transition metal ions was measured by the method of Price et al with some modifications.

Entrapment of Reactive Carbonyl Compounds
Two α-dicarbonyl compounds, ie, glyoxal (Sigma) and methylglyoxal (Sigma), were used as carbonyl compounds. The entrapment of α-dicarbonyls of the tested compounds were measured as described previously.

Pyridoxal 5'-Phosphate Entrapment
The entrapment of pyridoxal 5'-phosphate of the tested compounds was assayed by reversed phase HPLC with a fluorescence detector at excitation-emission wavelength of 300/400 nm.

Radioligand Binding Assay
Cell membranes (5 μg/well) from Chinese hamster ovary (CHO) cells overexpressing AT1R were incubated at room temperature for 120 minutes with 0.1 nmol/L radiolabeled [125I] Tyr4-angiotensin II in the presence of various concentrations of tested compounds. The affinity of the tested compounds for the AT1R was then assessed by a kit (CytoTools: PerkinElmer) with slight modifications.

The affinity of tested compounds for the AT2R was also examined by the same method as that of the AT1R affinity measurement, except for the use of membranes (2 μg/well) from Hela cells overexpressing AT2R.

Animal Studies
All animal experiments were performed in accordance with the guidelines of the Committee on Ethical Animal Care and Use of Tokai University, or with the Animal Experimentation Guidelines of Daiichi-Sankyo Co Ltd.

Type 2 Diabetic Rat Models With Nephropathy
The renoprotective effect of R-147176 was evaluated in an obese hypertensive type 2 diabetic rat model, ie, spontaneously hypertensive/NIH-corpulent (SHR/NDmcr-cp) rats, and in another normotensive type 2 diabetic rat model, ie, Zucker diabetic fatty (ZDF) rats.

Ten male SHR/NDmcr-cp rats, purchased from SLC (Shizuoka, Japan), were randomly divided into 2 groups: rats on vehicle (SHR/ND+vehicle, n = 5) and rats on R-147176 (30 mg/kg/d, orally) (SHR/ND+R, n = 5). Drug treatment was initiated at the age of 13 weeks and continued for 20 weeks.

Fifteen male ZDF rats (ZDF/Gmi-fa/fa), purchased from Charles River Japan, were randomly divided into 2 groups: rats on vehicle (ZDF+vehicle, n = 7) and rats on R-147176 (50 mg/kg/d, orally; ZDF+R, n = 8). Drug treatment was initiated at the age of 17 weeks and continued for 20 weeks.

Remnant Kidney Rat Model
The renoprotective effect of R-147176 was evaluated in the remnant kidney (RK) rat model. Fifteen male Wistar rats, purchased from Charles River, Japan, underwent subtotal nephrectomy after 1 week of acclimatization: via a midline incision and under 50 mg/kg ketamine anesthesia, the right kidney was removed and the posterior and 1 or 2 of the anterior branches of the left renal artery ligated. After 3 days, they were divided into 2 groups: rats on vehicle (RK+vehicle, n = 6) and rats on R-147176 (30 mg/kg/d orally; RK+R, n = 9). Drug treatment was continued for 4 weeks. In all in vivo rat studies, systolic blood pressure was determined at 2 and 4 weeks after the initiation of therapy.

Figure 1. Chemical structure of R-147176. Molecular weight, 380.46.

Renal Pentosidine Content
The pentosidine content of the kidney was measured as described previously.

Histological Evaluation of Glomerular Damage
Kidney sections (4-μm thickness) were fixed in methyl-Carnoy solution and stained with periodic acid-Schiff (PAS) or fixed in 10% formalin solution and stained with periodic acid-methenamine-silver (PAM). They were examined by light microscopy in a blinded fashion. Glomerular sclerosis was semiquantitatively evaluated by a previously described method.

Statistical Analysis
All data are reported as the mean±SE. Statistical analysis was performed with SPSS for Windows version 15.0 (SPSS). Comparisons between 2 groups were performed using an unpaired t test. For multiple comparisons, 1-way analysis of variance (ANOVA) and Tukey post hoc test were performed. P<0.05 was considered significant.

Results
Development of a Novel Synthetic Compound, R-147176
A total of 139 newly synthesized imidazole compounds derived from ARBs were assayed in vitro for AGE inhibition. The 9 most potent AGE inhibitors were then evaluated for transition metal chelation, AT1R affinity, and pharmacokinetic parameters. Eventually, R-147176 was identified as the most promising compound with a high AGE inhibitory effect and a low AT1R affinity. Its chemical structure (Figure 1) contains an imidazo-pyridine ring which is a part of the potent ARB, L-158, 809. R-147176 was used in subsequent studies.

In Vitro Characteristics of R-147176
R-147176 inhibited in vitro the production of pentosidine in uremic (Table 1) as well as in nonuremic diabetic plasma (data not shown). As shown by its IC50, R-147176 is more effective than previously reported AGE inhibitors, ie, aminoguanidine, pyridoxamine, and losartan. Unlike aminoguanidine and pyridoxamine, R-147176 just as the 2 other listed ARBs, chelated transition metal ions involved in the Fenton reaction that generates hydroxyl radicals (Table 1). Similarly, unlike aminoguanidine but like olmesartan, R-147176 failed to trap reactive carbonyl compounds precursors (glyoxal and methylglyoxal) for AGEs as well as pyridoxal 5'-phosphate (see supplemental Figure 1).
Table 1. Half-Maximal Inhibition (IC50) Value of Test Compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Pentosidine (mmol/L)</th>
<th>Transition Metal Chelating (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olmesartan</td>
<td>2.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Losartan</td>
<td>5.9</td>
<td>1</td>
</tr>
<tr>
<td>R-147176</td>
<td>2.2</td>
<td>3.3</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>6.8</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>6.1</td>
<td>1380</td>
</tr>
</tbody>
</table>

Pooled plasma from hemodialysis patients was incubated with the tested reagents (final concentration of 0.8, 2.0, and 5.0 mmol/L) for 1 week under air at 37°C. The pentosidine content was analyzed on a reverse-phase HPLC. The chelating activity of the tested compounds for transition metal ions was measured by the method of Price et al with some modifications. Ascorbic acid (500 μmol/L) was incubated at 30°C with 500 nmol/L CuCl2 in 50 mmol/L phosphate buffer (pH 7.4) in the presence of several concentrations of tested compounds. The ascorbic acid content of the incubation mixtures as determined by HPLC and the concentration required for a 50% inhibition (IC50) of the copper-catalyzed rate of ascorbic acid autoxidation in phosphate buffer was evaluated.

The inhibition of 125I-angiotensin II binding to the human AT1R by R-147176 was minimal when compared to reference angiotensin II receptor antagonists (Table 2). Based on KI values, R-147176 was 6700 times less effective than olmesartan in AT1R binding inhibition. R-147176 had no binding affinity to the human AT2R: IC50 values of R-147176 and PD-123319 (AT2-selective inhibitor) were >10 μmol/L and 7.5 nmol/L, respectively.

Toxicity and Pharmacokinetics of R-147176
Cellular injury was assessed in vitro in HeLa cells as the LDH activity released into the culture medium after 24 hours. Toxicity and Pharmacokinetics of R-147176

Blood Pressure Effect of R-147176 in Normotensive Rats
R-147176 at a dose of 10 and 50 mg/kg/d did not lower systolic blood pressure in normal rats (110±3 mm Hg and 111±7 mm Hg, respectively) in comparison with vehicle (119±6 mm Hg). By contrast, olmesartan (5 mg/kg/d) significantly decreased systolic blood pressure (87±3 mm Hg; P<0.01).

Type 2 Diabetic Rat Model, SHR/NDmcrcp
Data on experimental animals at the end of the study are summarized in supplemental Table I. SHR/NDmcrcp rats given vehicle exhibited hypertension and a metabolic syndrome derived from hyperphagia, ie, obesity, hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hypertriglyceridemia. R-147176 (30 mg/kg/d for 20 weeks) increased body weight but failed to modify the other parameters, including hypertension (Figure 2A).

SHR/NDmcrcp rats developed diabetic nephropathy with severe proteinuria (Figure 2B). The progressive rise in proteinuria observed in rats given vehicle was lowered throughout the study by R-147176 (Figure 2B). At the end of the study, proteinuria reached 195.2±28.4 mg/d in the vehicle group (n=5) and 91.8±21.7 mg/d in the R-147176 treated group (n=5, P<0.05).

The renal contents of pentosidine, taken as a marker of AGEs, was determined at the end of the study (Figure 2C). R-147176 significantly decreased (P<0.05) renal pentosidine from 0.149±0.009 pmol/mg in rats treated with vehicle to 0.099±0.013 pmol/mg in rats given R-147176.

Further chemical analyses revealed that R-147176 significantly (P<0.05) decreased renal contents of Nε-carboxymethyllysine (CML), another AGE moiety, from 77.0±2.4 ng/mg in vehicle-treated rats to 69.9±2.1 ng/mg in R-147176-treated rats and of nitrotyrosine, a nitrosative stress product, from 0.990±0.16 pmol/mg in vehicle-treated rats to 0.33±0.11 pmol/mg in R-147176-treated rats. It also decreased, though not significantly (P=0.0759), renal protein carbonyls, an oxidative protein product, from 2.08±0.55 in vehicle-treated rats to 0.76±0.27 in R-147176-treated rats, expressed as density arbitrary unit.

Representative PAS-stained renal pictures obtained at the end of the study in each SHR/NDmcrcp animal group are shown in Figure 2D and 2E. Focal and segmental sclerosis observed in rats given vehicle was reduced in rats given R-147176. The glomerular injury score fell from 0.77±0.08 in rats on vehicle to 0.50±0.07 in rats on R-147176 (P<0.05, Figure 2F).

We further evaluated, by immunohistochemical ED-1 staining, the degree of macrophage infiltration in the kidney of SHR/NDmcrcp rats. ED-1 staining was significantly (P<0.01) decreased by R-147176 (infiltration score fell from 38.4±3.8 in rats on vehicle to 19.9±1.5 in rats on R-147176).

Type 2 Diabetic Rat Model, ZDF
ZDF rats developed severe diabetes with hyperglycemia and hypercholesterolemia, a moderate hypertension (see supplemental Table II), and a loss of body weight. R-147176, at a
dose of 50 mg/kg/d, lowered mildly but significantly, systolic blood pressure (Figure 3A) and markedly hypercholesterolemia, but failed to modify body weight or hyperglycemia.

ZDF rats developed diabetic nephropathy with severe proteinuria (Figure 3B). The progressive rise in proteinuria observed in rats given vehicle was lowered throughout the study by R-147176. At 20 weeks, proteinuria reached $309.7 \pm 23.0$ mg/d in the vehicle group ($n = 7$) versus $107.7 \pm 23.0$ mg/d in the R-147176 group ($n = 8$, $P < 0.01$).

Representative PAM-stained renal pictures obtained at the end of the study in each ZDF animal group are shown in Figure 3C and 3D. Focal and segmental sclerosis observed in rats given vehicle was improved in rats given R-147176. The glomerular injury score fell from $0.53 \pm 0.09$ in rats on vehicle to $0.32 \pm 0.06$ in rats on R-147176, but this difference did not reach statistical significance ($P = 0.09$, Figure 3E).

**Remnant Kidney Rat Model**

Rats with a remnant kidney did not gain weight or develop proteinuria but became slightly hypertensive ($n = 6$). R-147176 at a dosage of 30 mg/kg/d ($n = 9$) did not modify body weight or blood pressure (Figure 4A). At the end of the study, urinary protein excretion averaged $50.9 \pm 14.4$ mg/d and $25.2 \pm 4.1$ mg/d in rats given vehicle or R-147176, respectively ($P = 0.06$).

Representative PAS-stained pictures obtained at the end of the study in each animal group are shown in Figure 4B and 4C. Focal segmental or global sclerosis present in the vehicle-treated group were markedly improved by R-147176. The glomerular injury scores fell from $1.7 \pm 0.3$ in rats on vehicle to $0.6 \pm 0.2$ in rats on R-147176 ($P < 0.01$, Figure 4D). An improvement of macrophage infiltration was observed in the remnant kidney model ($P < 0.001$): infiltration score fell from $44.5 \pm 3.4$ in rats on vehicle to $21.6 \pm 1.8$ in rats on R-147176.

**Discussion**

The present data demonstrate that a novel ARB-derivative, endowed with a potent AGE inhibitory activity, reduces proteinuria and protects the kidney, despite a minimal affinity for the AT1R and thus virtually no antihypotensive effect. These observations cast a new light on the mechanisms whereby ARBs protect the kidney as demonstrated in experimental models and clinical conditions, paramount among which diabetic nephropathy.¹⁻⁴ Experimental and clinical evidence disclosed that blood pressure changes are not the sole mediators of these renal benefits.⁵⁻⁸ The present study now suggest that, at least in experimental diabetic nephropathy as well as in remnant kidney disease, such a role is at best only marginal as shown by the antiproteinuric and renoprotective action of R-147176 despite the stability of blood pressure. It further indicates that interference with AT1R activity is not implicated. By contrast, it establishes that the main renal impact of ARBs relates to the inhibition of advanced glycation and oxidative stress as shown by the in vitro studies as well as by the in vivo documented reduction of the renal pentosidine, CML and nitrotyrosine contents. These conclusions identify clearly advanced glycation and

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Figure 2. Effect of R-147176 in SHR/NDmc-cp rats. Rats were given vehicle (filled triangle) or 30 mg/kg/d R-147176 (open triangle). A, Systolic blood pressure; B, Urinary protein excretion; C, Renal pentosidine content; D and E, PAS-stained renal tissues (Magnification, ×400); F, Glomerular sclerosis score. * $P < 0.05$, ** $P < 0.01$ vs vehicle.
oxidative stress as therapeutic targets for the prevention and treatment not only of diabetic but also of other nephropathies exemplified here by the remnant kidney rat model.

Interestingly, 2 previous studies\textsuperscript{11,12} have used the same protocol in the SHR/NDmc-cp diabetic rat strain to ascertain the protective effect of 5 mg/kg olmesartan, an antihypertensive AT1R inhibitor. Systolic blood pressure fell by 31\% (30.6 and 30.7), proteinuria by 55.6\% (57.2 and 54.0), and renal pentosidine content by 51.5\% (42.6 and 60.3). These data are to be compared with the present ones: although systolic blood pressure did not fall significantly (–7.9\%), proteinuria decreased by 53\% and renal pentosidine content by 33.6\%. Although collected at different times in the past, these results strongly support our conclusion that most, if not all, the protective effects of ARBs on the kidney are independent of blood pressure changes but rely on the inhibition of advanced glycation and oxidative stress.

Although the present study does not completely rule out a minor role of AT1R inhibition in the renoprotective effect of ARBs, it should be kept in mind that R-147176 is 6700 times less effective than olmesartan in AT1R binding and that blood pressure changes were minimal.

The mechanism of the AGE inhibitory effect of R-147176 clearly differs from that of classical AGE inhibitors.\textsuperscript{10,12} Aminoguanidine\textsuperscript{10} and OPB-9195,\textsuperscript{20} i.e., hydrazine or guanidine derivatives, inhibit AGE formation by trapping their reactive carbonyl precursors as well as pyridoxal, the latter effect precluding their long-term use in man. Pyridoxamine\textsuperscript{21} as well as the recently reported LR-90\textsuperscript{22} also traps reactive carbonyl precursors. By contrast, R-147176 inhibits advanced glycation through the previously described mechanisms of ARBs\textsuperscript{10}: it does not trap reactive carbonyl precursors but reduces their production through an inhibition of oxidative stress (i.e., hydroxyl radicals scavenging and inhibition of the Fenton reaction).\textsuperscript{10} Unlike ARBs, however, it does not bind strongly to the human AT1R in vitro and has therefore no comparable effect on blood pressure in vivo.

R-147176 might be relevant as a therapeutic agent in human disease. It is orally bioavailable as demonstrated by its pharmacokinetics. It is toxicologically safe. In vitro, its cytotoxicity is very low. In both mice and rats single doses of up to 2.0 g/kg at one time proved nontoxic. In rats, R-147176 (200 mg/kg/d) given daily for 2 weeks did not result in observable subacute toxicity including hyperkalemia. Whether any longer-term toxicity exists remains to be seen in future studies.

The minimal effect of R-147176 on blood pressure might prove helpful in normotensive or hypotensive patients in whom an inappropriate hypotension is contraindicated. For example, the eventual consequences of stroke are related not only to the infarct size but also to numerous factors among which the prevailing level of blood pressure. An ARB-induced blood pressure reduction occasionally decreases cerebral blood supply to the penumbral region, impacting on mortality and disabling long-term morbidity.\textsuperscript{23–26} R-147176 should thus expand our therapeutic options in the treatment of cardiovascular and kidney diseases.

In conclusion, we have synthesized a novel, nontoxic, ARB-derivative, R-147176, with an extremely weak affinity for the AT1R but a powerful inhibitory effect on oxidative stress and advanced glycation. It displays renal protective characteristics without influencing blood pressure opening new avenues to treat normotensive or hypotensive patients with cardiovascular diseases including kidney disease. The dissociation between renoprotection and blood pressure changes strongly suggest that ARBs act through their inhibitory effect on AGEs and oxidative stress.
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Disclosures
None.

References


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Supplement to the article by Izuhara et al “A novel sartan derivative with very low angiotensin II type 1 receptor affinity protects the kidney in type 2 diabetic rats”

Methods

Reagents

Olmesartan (R & D Division of Daiichi-Sankyo Co., Ltd.), aminoguanidine hydrochloride (Tokyo Chemical Industry, Tokyo, Japan), pyridoxamine (Sigma, St. Louis, MO) and losartan potassium (Wako, Osaka, Japan) were kindly provided or purchased.

All tested compounds were dissolved in DMSO to obtain a stock solution of 50 mM to be further diluted to obtain the required concentration (the final concentrations of DMSO were less than 10%). We have confirmed that DMSO, at the final concentration of 10%, does not influence the used assays.

Renal Nε-carboxymethyllysine and nitrotyrosine contents

Kidney tissue was homogenized in phosphate buffered saline and centrifuged at 4°C. Nε-carboxymethyllysine (CML) and nitrotyrosine contents of the supernatants was quantified by competitive and sandwich ELISA (CML, FUSHIMI Pharmaceutical Co., Ltd., Kagawa, Japan; nitrotyrosine, Northwest Life Science Specialties, LLC, WA, USA), respectively.
**Protein carbonyls**

Proteins extracted from whole kidney were separated in a 4-20 % SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. Western blot analysis for protein carbonyls was performed with the OxyBlot protein oxidation detection kit (Millipore, MA, USA). Blots were visualized using the ECL plus Western blotting detection system (Amersham Biosciences, Piscataway, USA). The density of the bands was measured by a computer software (ATTO Co., Tokyo, Japan).

**Immunohistochemistry**

Indirect immunoperoxidase methods were used to evaluate the monocytes/macrophages infiltration with a mouse monoclonal antibody ED-1 (Serotec, Oxford, UK). ED-1-positive cells were counted in 20 randomly selected cortical fields with an x20 objective in a blinded manner.

**Radioligand binding assay**

The affinity of tested compounds for the AT1R was assessed by a kit (CytoTools™; PerkinElmer) with slight modifications (1). Membranes (5 µg/well) from Chinese hamster ovary (CHO) cells overexpressing AT1R were incubated at room temperature for 120 min with 0.1 nM radiolabeled $[^{125}I]$ Tyr$^4$-angiotensin II (PerkinElmer) in the presence of various concentrations of tested compounds in a buffer solution containing 40 mM Tris-HCl, 240 mM NaCl, 10 mM MgCl$_2$, 0.9 mM EDTA and 0.04 % bovine serum albumin (pH 7.4). The reaction was terminated by a rapid filtration of the
incubation solution through glass fiber filters (Whatman GF/B filters) presoaked with 0.3 % polyethylilenimine in water. The filters were washed 3 times with ice-cold 20mM Tris-HCl buffer (pH 7.4). The radioactivity trapped on the filter was determined by a gamma-counter (Packard). The specific binding was defined as the difference between the total binding and the non-specific binding in the presence of 10 µM unlabeled angiotensin II. The inhibition constant (Ki) values were calculated from the respective IC₅₀ (the concentration of the drug required for 50% inhibition) values using the following formula: 

\[ K_i = \frac{IC_{50}}{1+L/K_d} \]

where L is the concentration of the radiolabel ligand and K_d is its dissociation constant obtained from Scatchard analysis.

The affinity of tested compounds for the AT2R was also assessed by the same method except for the usage of membranes (2 µg/well) from Hela cells overexpressing AT2R.

*In vitro toxicity*

HeLa cells were cultured for 24 hrs in the DMEM in the presence of the tested compound (50-150 µM). Cytotoxicity was determined by the release of lactate dehydrogenase (LDH) in the culture medium measured with a kit (Promega, Madison, WI) and expressed as percentage of the total LDH activity released after freeze and thaw lysis of all culture cells.

*Animal studies*

All animal experiments were performed in accordance with the guidelines of the
Committee on Ethical Animal Care and Use of Tokai University, or with the Animal Experimentation Guidelines of Daiichi-Sankyo Co., Ltd.

Systolic blood pressure was determined in conscious rats by the tail-cuff method. Rats were housed in metabolic cages for overnight collection of urine. Total cholesterol, triglycerides, and glucose concentrations in plasma and protein concentration in urine were determined with an automatic analyzer (Hitachi Automatic Clinical Analyzer 7170, Hitachi Science Systems, Ibaraki, Japan). Plasma insulin was measured with a commercially available kit (Morinaga Biochemistry Lab, Tokyo, Japan). HbA1c was measured using the DCA2000 (Bayer Diagnostics, Pittsburgh, PA).

**Blood pressure lowering effect in normal rats**

Twenty-one male SD rats weighing 290-320 g, purchased from Charles River Japan, were randomly divided into five groups: rats on vehicle (n = 6), rats on R-147176 (10 or 50 mg/kg/day, orally) (each n = 5), and rats on olmesartan (5 mg/kg/day, n = 5). Drug treatment was continued for 5 days. Systolic blood pressure was determined at day 5.

**Acute and sub-acute toxicities**

For *in vivo* acute toxicity studies, R-147176 was given by oral gavage as a single dose (500, 1000 and 2000 mg/kg) to ICR mice weighing 30-35 g (CLEA Japan, Inc., Tokyo, Japan) and also administrated as a single dose (300, 1000 and 2000 mg/kg) to SD rats weighing 180-200 g (Charles River Japan, Inc., Tokyo, Japan). The toxic consequences were monitored for 2 and 1 weeks in mice or rats respectively. At the end of study, each
mouse or rat was autopsied and various organs evaluated.

For *in vivo* sub-acute toxicity studies, R-147176 (200 mg/kg, once a day) was also given by oral gavage to SD rats for 2 weeks. The animals were weighed and evaluated at the end of the study,

*Pharmacokinetics study in normal rats*

R-147176 (50 mg/kg) was administered by oral gavage to male Wistar rats weighing 180-200 g (Charles River Japan). Heparinized blood samples were collected from the vein before (0 h) and 1, 2, 6 and 24 h after administration. Plasma concentration was analyzed by reverse-phase HPLC. Maximum drug concentration time (Tmax), maximum drug concentration (Cmax), and drug half-life (T1/2) were calculated.
Reference

**Tables**

**Table I.** Physiological and biochemical data of the obese, hypertensive, diabetic rats (SHR/NDmcr-cp) at the end of the study (aged 33 weeks).

<table>
<thead>
<tr>
<th></th>
<th>SHR/ND + vehicle</th>
<th>SHR/ND + R-147176</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>711 ± 7</td>
<td>753 ± 10*</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>192.2 ± 6.6</td>
<td>177.1 ± 6.8</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>4.2 ± 0.4</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td><strong>Total cholesterol (mg/dl)</strong></td>
<td>187 ± 9</td>
<td>171 ± 12</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dl)</strong></td>
<td>740 ± 60</td>
<td>613 ± 198</td>
</tr>
<tr>
<td><strong>Insulin (ng/ml)</strong></td>
<td>60 ± 38</td>
<td>101 ± 79</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE.

*P < 0.05 vs. SHR/ND + vehicle
### Table II. Physiological and biochemical data of the obese, hypertensive, diabetic rats (ZDF) at the end of the study (aged 37 weeks).

<table>
<thead>
<tr>
<th></th>
<th>ZDF + vehicle</th>
<th>ZDF + R-147176</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>367 ± 24</td>
<td>314 ± 9</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>138.4 ± 6.1</td>
<td>119.3 ± 4.4*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>514 ± 46</td>
<td>542 ± 42</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>298 ± 48</td>
<td>164 ± 14*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE.

*P < 0.05 vs. ZDF + vehicle
**Figure**

**Figure I.** Entrapment of reactive carbonyl compounds (RCOs) and pyridoxal 5'-phosphate. The residual glyoxal (A), methylglyoxal (B) and pyridoxal 5'-phosphate (C) were determined by HPLC. Data are expressed as mean ± SE.