A Highly Potent and Long-Acting Oral Inhibitor of Human Renin

Kunio Hiwada, Tatsuo Kokubu, Eiki Murakami, Shinjiro Muneta, Yasuhiro Morisawa, Yuichiro Yabe, Hiroyuki Koike, and Yasuteru Iijima

SUMMARY An orally active renin inhibitor, ES 6864 (N-[(2R)-3-morpholinocarbonyl-2-(1-naphthylmethyl)propionyl]-(4-thiazolyl)-L-alanyl-cyclostatine-(2-morpholinoethyl)amide), was synthesized. ES 6864 was found to be a highly potent inhibitor of human renin with a $K_i$ value of $7.3 \times 10^{-9}$ M. The compound competitively inhibited human renin. The inhibitor was also potent against monkey renin but was less effective against renins from pig, dog, goat, rabbit, and rat. ES 6864 did not inhibit cathepsin D, pepsin, trypsin, chymotrypsin, angiotensin converting enzyme, and urinary kallikrein at a concentration of $10^{-5}$ M. ES 6864 was resistant to proteolytic actions of the enzymes in rat tissue homogenates (liver, kidney, pancreas, and small intestine). Oral administration of ES 6864 at 30 mg/kg to conscious, sodium-depleted marmosets produced a significant blood pressure reduction and almost complete inhibition of plasma renin activity, which persisted for 5 hours. Oral administration of ES 6864 also produced dose-related decreases of blood pressure in hog renin-infused rats, but the duration of action was much shorter than that in conscious marmosets. The parent compound in the blood following oral administration of ES 6864 to marmosets was confirmed directly by measuring the plasma concentration of ES 6864. These results enhance the possibility of developing renin inhibitors that can be used clinically. (Hypertension 11: 708-712, 1988)

Key Words • human renin inhibitor • competitive inhibition • oral administration • marmoset • hog renin-infused rat • plasma renin activity • blood pressure

Renin (EC 3.4.99.19) cleaves angiotensinogen to release angiotensin I (Ang I), which in turn is converted to the active peptide angiotensin II by angiotensin converting enzyme (ACE; EC 3.4.15.1). Inhibition of the initial step in the renin-angiotensin system is an attractive prospect because ACE inhibitors are widely used for the treatment of hypertensive patients. Several novel renin inhibitors with inhibitory potency sufficient for in vivo use have been synthesized, but no effective compound is yet available for use as an antihypertensive drug based on renin inhibition. The major problem has been the lack of oral activity and insufficient duration of action; renin specificity of the inhibitor is an additional obstacle. Our previous articles have dealt with highly potent inhibitors of human renin that are small peptide analogues containing statine and that are poorly absorbed. The present report describes an orally active renin inhibitor containing statine analogue, ES 6864, (N-[(2R)-3-morpholinocarbonyl-2-(1-naphthylmethyl)propionyl]-(4-thiazolyl)-L-alanyl-cyclostatine-(2-morpholinoethyl)amide). This compound is highly potent, renin-specific, and long-acting in vivo.

Materials and Methods

[125I]Ang I was purchased from New England Nuclear (Boston, MA, USA). Ang I, hippuryl-L-histidyl-L-leucine, N-benzoyl-L-arginine 4-nitroanilide HCl, and pepstatin A were obtained from the Peptide Institute (Minoh, Osaka, Japan). N-Succinyl-L-phenylalanine 4-nitroanilide and hog angiotensinogen (2.0 U/mg of protein) were purchased from Sigma Chemical (St. Louis, MO, USA). D-Valyl-L-leucyl-L-arginine 4-nitroanilide 4-nitroanilide and hog angiotensinogen (2.0 U/mg of protein) were purchased from Sigma Chemical (St. Louis, MO, USA). D-Valyl-L-leucyl-L-arginine 4-nitroanilide 4-nitroanilide and hog angiotensinogen (2.0 U/mg of protein) were purchased from Sigma Chemical (St. Louis, MO, USA).
troanilide 2 HCl was obtained from Kabi Diagnostica (Stockholm, Sweden). Alfadione (Saffan, Glaxo), used for the anesthesia of the marmoset, was kindly supplied by Dr. Wood (CIBA-Geigy, Basel, Switzerland). Marmosets (Callithrix jacus; 12–18 months old) were purchased from Charles River (Margate, Kent, UK).

Human renal renin, prepared by a method described previously,13 contained a specific activity of 40 µg Ang I/mg of protein/hr. Animal renins and human, sheep, rabbit, and rat angiotensinogens used in this study have been described previously.12,13 Human plasma with high renin activity (>5 ng Ang I/ml/hr) was collected from patients with various disorders and was pooled. Preparations of purified human kidney ACE and partially purified human urinary kalikrein have been described previously.13

In Vitro Inhibition Experiments
ES 6864 was dissolved in 30% ethyl alcohol. Renin activities in the presence and absence of the inhibitor were measured with the corresponding angiotensinogens in 0.1 M phosphate buffer (pH 7.3) containing angiotensinase inhibitors (10 mM EDTA and 3.4 mM 8-hydroxyquinoline) at 37°C for 10 minutes. The amount of Ang I generated was measured by radioimmunoassay.13 The inhibition constant (Kᵢ) for ES 6864 was determined using the plot of Dixon and Webb.16 Human plasma renin activity (PRA) and marmoset PRA were measured by a method described previously.12,13 Cathepsin D (bovine spleen; 13 U/mg of protein), chymotrypsin (bovine pancreas; Sigma), ACE (human), and urinary kalikrein (human) were measured in the presence and absence of ES 6864 with the corresponding substrates as described previously.13 Protein was measured by the method of Lowry et al.,17 with bovine serum albumin used as the standard.

In Vitro Metabolic Stability
Fresh liver, kidney, pancreas, and small intestine from a Sprague-Dawley rat were homogenized in 10 volumes (wt/vol) of 0.2 M phosphate-buffered saline, pH 7.2, and centrifuged at 2000 rpm for 20 minutes. ES 6864 dissolved in a small amount of 0.1 M citric acid was diluted with 0.2 M phosphate-buffered saline to a 10 µM solution. The solution of ES 6864 (0.4 ml) and phosphate-buffered saline containing 12.5 mM MgCl₂ (0.4 ml) was added to 0.2 ml of the tissue homogenates and incubated at 37°C. The enzyme reaction was terminated by adding 4 ml of methanol at different times. The amount of ES 6864 in the supernatant solution was measured by the method described in the following section.

Measurement of Plasma Concentration of ES 6864
To 50 µl of plasma was added 200 µl of methanol, and the mixture was vortexed and centrifuged. After the supernatant was evaporated to dryness, the residue was redissolved in 50 µl of 50% ethanol. An aliquot in a volume of 20 µl was applied to a high performance liquid chromatography column packed with octadecylsilane (YMC-ODS-A312, Yamamura Kagaku, Osaka, Japan). The column was eluted with a mixture of acetonitrile and 50 mM potassium dihydrogen phosphate solution (45:55). The effluent was monitored with a fluorescence detector (excitation wavelength of 228 nm; emission wavelength of 335 nm).

In Vivo Evaluation in Marmosets
Marmosets of either sex weighing 280 to 350 g were fed a low sodium diet for 1 week. With the marmosets under anesthesia (alfadione), a polyethylene catheter (PE-10) was placed into the left femoral artery. The other end of the catheter was led under the skin and exteriorized at the root of the tail. On the next day (i.e., at least 15 hours later), the animal was placed in a plastic cage and the femoral catheter was connected to a pressure transducer to measure blood pressure and heart rate (Lineacorder WR3701, Graphtec, Tokyo, Japan). After blood pressure and heart rate were stabilized, a 0.2-ml blood sample was drawn from the femoral catheter and the same amount of saline was infused. ES 6864 was dissolved in 0.1 M citric acid and was given orally in a volume of 2 ml/kg with an infant feeding tube. Blood sampling was repeated 1, 2, 3, and 5 hours after oral administration of the compound. The blood samples were assayed for PRA and plasma concentration of the compound.

In Vivo Evaluation in Renin-Infused Rats
Male Wistar rats (n = 14; Charles River Japan, Hino, Shiga, Japan) weighing about 200 g, which had been fasting for 24 hours, were anesthetized with pentobarbital sodium (50 mg/kg i.p.). An endotracheal tube was inserted, and bilateral vagotomy was performed. A carotid artery was cannulated with polyethylene tubing (PE-50) and connected to a chart recorder, as just described, through a transducer for the continuous recording of mean arterial blood pressure. A femoral vein was cannulated with the same type of catheter and connected to a Harvard infusion pump (South Natick, MA, USA) for the intravenous administration of hog renin. Following surgical preparation, ganglion blockade was accomplished with pentriniurn (0.1 mg/kg s.c.). After the blood pressure had stabilized, partially purified hog renin (specific activity assayed with rat angiotensinogen, 2.7 µg Ang I equivalents/mg of protein/min) was infused at a rate of 4 ng of angiotensin II equivalents/min, which induced an elevation of 60 ± 5 mm Hg in mean arterial blood pressure. The reactivity to angiotensin II did not change for at least 5 hours in the rat preparations. After the elevated blood pressure had restabilized, ES 6864 dissolved in 0.1 M citric acid (1.0 ml) was administered into the stomach using an infant feeding tube. The data are presented as percent changes of inhibition of the renin-induced blood pressure elevation; 100% inhibition corresponded to the blood pressure observed after vagotomy and ganglion blockade but before hog renin infusion.
Statistical Analysis
Data are expressed as means ± SEM. Statistical analysis was performed using Student's paired t test.

Results
In Vitro Inhibitory Potency of ES 6864
The compound ES 6864 did not cross-react with Ang I antibody at a concentration of 10^-4 M. Pepstatin A, which was used as a standard renin inhibitor, inhibited human renin with a 50% inhibitory concentration (IC_{50}) of 3.6 × 10^{-5} M at pH 7.3. ES 6864 inhibited human renin with an IC_{50} of 7.0 × 10^{-9} M. The IC_{50} value of ES 6864 for human renin and human angiotensinogen was 7.3 × 10^{-9} M. The mode of inhibition proved competitive. The compound also inhibited human plasma renin by 50% at 6.9 × 10^{-9} M.

The inhibitory effects of ES 6864 were studied in renins from six different species of animals (Table 1). The human renin inhibitor was similarly potent against monkey renin but was one or two orders of magnitudes less active against renins from dog, rabbit, goat, and pig. The inhibitor was very weak against rat renin.

The inhibitory effects of ES 6864 on aspartyl proteases, such as cathepsin D and pepsin, and other proteases were also studied. The compound did not inhibit cathepsin D, pepsin, trypsin, chymotrypsin, ACE, and urinary kallikrein at a concentration of 10^{-5} M.

Metabolic Stability of ES 6864 in Rat Tissue Homogenate
The metabolic stability of ES 6864 in rat tissue homogenates was evaluated. ES 6864 was not degraded by kidney, pancreas, and intestinal homogenates, but it was degraded by about 20% by liver homogenate in the 1-hour incubation period.

Absorption from Gastrointestinal Tract
Plasma concentrations of ES 6864 were measured in conscious marmosets after oral administration of the compound (3, 10, and 30 mg/kg). The dose-response relationship was observed at doses of 10 and 30 mg/kg. Figure 1 shows plasma concentrations of ES 6864 in two marmosets given 30 mg/kg of the compound by oral administration. Plasma concentration of ES 6864 reached a maximum of 1.2 μg/ml at 1 hour and decreased rapidly. The rapid decrease in the plasma concentration of ES 6864 was followed by a slow decay phase between 3 and 8 hours. ES 6864 was not detected in plasma when administered orally at 3 mg/kg.

In Vivo Evaluation of ES 6864 in Marmosets
A single oral administration of ES 6864 at a dose of 3 mg/kg produced 67% inhibition of PRA at 1 hour, an effect that persisted for 5 hours. One and 5 hours after oral administration of 10 mg/kg of ES 6864, PRA was inhibited by 97 and 90%, respectively. Full inhibition was achieved at 30 mg/kg in the 5-hour observation period (Figure 2, upper panel). Mean blood pressure tended to decrease at ES 6864 doses of 3 and 10 mg/kg. At a dose of 30 mg/kg, mean blood pressure decreased significantly, reaching a nadir 2 to 3 hours after oral administration of the compound. Mean blood pressure at the nadir was 88.4 ± 2.5 mm Hg compared with

<table>
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<tr>
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<tr>
<td>Human (plasma)</td>
<td>Endogenous</td>
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<tr>
<td>Monkey</td>
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Each value is the mean of three measurements.

![Figure 1. Plasma concentration of ES 6864 after a single oral administration of the compound in two conscious marmosets. Each point represents the mean value.](image1)

![Figure 2. PRA and mean arterial blood pressure (MBP) as a function of time and of the oral dose of ES 6864, 3 (•; n = 4), 10 (■; n = 4), and 30 mg/kg (●; n = 5), or of vehicle (Ο; n = 3) in sodium-depleted conscious marmosets. Values are means ± SEM (bars). Asterisk indicates a significant reduction of MBP compared with the pretreatment value (p<0.05).](image2)
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113.2 ± 2.5 mm Hg before oral administration. Mean blood pressure remained lowered for 5 hours (see Figure 2, lower panel). There was no significant change in heart rate. The vehicle (0.1 M citric acid) did not affect the blood pressure and heart rate.

In Vivo Evaluation of ES 6864 in Renin-Infused Rats

Figure 3 shows the results of oral administration of ES 6864 in anesthetized, ganglion-blocked, and hog renin-infused rats. The magnitude of hog renin-dependent blood pressure elevation was 60 ± 5 mm Hg in the rat preparation. The vehicle (0.1 M citric acid) had no significant effect on the elevated blood pressure. Oral administration of ES 6864 at doses of 50, 200, and 500 mg/kg produced dose-dependent decreases in mean blood pressure. The hypotensive effect was apparent 5 minutes after oral administration and reached a nadir at about 30 minutes. At a dose of 500 mg/kg, ES 6864 maximally inhibited about 50% of the renin-dependent blood pressure component; this value dropped to 30% 90 minutes after oral administration.

Discussion

The present study demonstrated that ES 6864 is a potent human renin inhibitor that is highly species-specific and enzyme-specific. These in vitro profiles are shared with another renin inhibitor (ES 1005) reported previously. ES 6864 was much better absorbed by the gastrointestinal tracts of marmosets and rats than was ES 1005. The N-terminus and C-terminus of ES 1005 were modified, based on the notion that the oral absorption of a compound depends on the molecular size, solubility, and stability in digestive organs. Replacement of one of the naphthyl residues at the N-terminus of ES 1005 with a morpholinocarbonyl group made the resulting molecule more hydrophilic. In addition, the presence of the morpholinoethyl group at the C-terminus of ES 6864 enhanced its solubility in water. ES 6864 contains no natural amino acid and is therefore resistant to proteolytic cleavage.

The results of a preliminary study in conscious marmosets showed that only a portion of the orally administered dose of ES 6864 reached the systemic circulation. Plasma concentration of ES 6864 was related to dose at doses of 10 mg/kg and more. The remainder probably underwent presystemic metabolism during the first passage through the liver. Although ES 6864 was not detected in plasma when administered orally at 3 mg/kg, PRA in marmosets was inhibited. This is due to the sensitivity of the measurement and may result from the metabolic changes of ES 6864 by the liver. We cannot detect the metabolites of ES 6864, which may have a renin inhibitory potency.

In vivo experiments in marmosets showed that orally administered ES 6864 produced a long-lasting inhibition of PRA. The compound inhibited PRA almost completely at doses over 10 mg/kg, and the inhibition lasted more than 5 hours. Although blood pressure tended to decrease at doses of 3 and 10 mg/kg, the blood pressure-lowering effect was not clear. ES 6864 lowered blood pressure significantly at a dose of 30 mg/kg 1 hour after oral administration, when plasma concentration of the compound exceeded 1 μg/ml. Blood pressure reached a nadir at 2 to 3 hours, when plasma concentration of ES 6864 was about half of the maximum concentration. There was a dissociation between the inhibition of PRA and the fall in blood pressure with respect to the dose and time course. Statine-containing renin inhibitory peptide showed a similar phenomenon. This phenomenon could be interpreted to indicate that the decreased level of tissue angiotensin II may not increase as rapidly as the inhibited PRA decreases.

Oral administration of ES 6864 to anesthetized, ganglion-blocked, and hog renin-infused rats resulted in a blood pressure reduction that was smaller in magnitude and shorter in duration than that elicited by U-71038 at the same dose. One possible explanation of the difference may be due to different inhibitory potencies of ES 6864 and U-71038 against hog renin. The duration of the hypotensive effect of orally administered ES 6864 was much longer in conscious marmosets than in hog renin-infused rats. The results suggest that not only PRA but also tissue renin in marmosets may be inhibited by ES 6864, while ES 6864 may inhibit only the exogenously infused hog renin in the preparations.

Recently, three orally active renin inhibitors have been reported. These compounds were tested in sodium-deficient marmosets, sodium-depleted monkeys, or hog renin-infused rats. The two compounds CGP 29287 (100 mg/kg) and U-71038 (50 mg/kg) almost completely inhibited PRA after oral administration, but the inhibition did not persist long. The in vivo inhibitory effect of our compound on PRA was stronger and lasted longer than those of the other two compounds. The long-lasting inhibition of PRA produced by ES 6864 is probably due to the metabolic stability of the compound.

In conclusion, our results demonstrate that ES 6864 is an oral renin inhibitor with high potency and specificity for human renin. The compound was resistant to

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**Figure 3.** Percent changes of mean arterial blood pressure (MBP) in anesthetized, ganglion-blocked, and hog renin-infused rats after a single oral administration of vehicle (○; n = 3) or ES 6864, 50 (▲; n = 2), 200 (■; n = 4), and 500 (▲; n = 5) mg/kg. Values are means ± SEM. Asterisk indicates a significant inhibition of the hog renin-dependent blood pressure elevation (p < 0.05).
proteolytic cleavage. A single oral administration of ES 6864 produced a long-lasting inhibition of PRA as well as blood pressure reduction in sodium-depleted marmosets. The duration of action of the compound was much longer than that reported for other renin inhibitors. The parent compound in the blood following oral administration was confirmed directly by measuring the plasma concentration of ES 6864. These results enhance the possibility of developing renin inhibitors that can be used clinically.

References
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