Highly Potent and Specific Inhibitors of Human Renin

TATSUO KOKUBU, KUNIO HIWADA, EIKI MURAKAMI, YOICHI IMAMURA, REI MATSUEDA, YUICHIRO YABE, HIROYUKI KOIKE, AND YASUTERU IIJIMA

SUMMARY Small peptide analogues representing the C-terminal portion of angiotensin I sequence were designed as inhibitors of human renin. Among synthesized compounds, benzyloxycarbonyl ("Z")-(1-naphthyl)Ala-His-leucinal (ES-188), Z-(1-naphthyl)Ala-His-statine ethyl ester (ES-226), and Z-(1-naphthyl)Ala-His-statine 2-methylbutylamide (ES-254) markedly inhibited human and primate renins (inhibitory concentration, 50% [IC50], near 10⁻⁷ M). These peptide analogues inhibited rabbit renin with one or two orders of magnitude less potency. They were very weak inhibitors of renins from pig, goat, dog, and rat. ES-188 had no discernible effect on cathepsin D, pepsin, or human angiotensin-converting enzyme at the concentration of 10⁻⁴ M. ES-226 had little effect on the three enzymes at the concentration of 10⁻⁵ M; however, ES-254 had a considerable inhibitory effect on cathepsin D (IC50 of 1.4 × 10⁻⁵ M), pepsin (IC50 of 4.2 × 10⁻⁵ M), and human angiotensin-converting enzyme (IC50 of 7.1 × 10⁻⁴ M). Our results indicate that 1-naphthylalanine-containing tripeptide analogues are highly potent human renin inhibitors. (Hypertension 7[Suppl I]: 1-8-1-11, 1985)

KEY WORDS • tripeptide analogues • 1-naphthylalanine • statine

RENIN (EC 3.4.23.15) acts on a protein substrate, angiotensinogen, to release the hemodynamically inactive angiotensin I (ANG I). The ANG I subsequently is converted to the potent pressor peptide angiotensin II (ANG II) by angiotensin-converting enzyme (ACE; EC 3.4.15.1). The development of orally active ACE inhibitors and the clear demonstration of their efficacy as antihypertensive drugs in patients with renin-dependent hypertension and essential hypertension have evoked the interest in the development of potent renin inhibitors that are orally active.

In 1968 we reported that methyl or ethyl ester of a tetrapeptide (Leu-Leu-Val-Tyr) in the sequence of equine angiotensinogen acted as a competitive inhibitor of rabbit renin. But the inhibitor constant was only in the millimolar range. To develop more potent inhibitors of renin, many analogues of a large segment of renin substrate subsequently have been synthesized, and inhibitory potency has been improved by replacing the peptide bond (—CO—NH—) at the cleavage site of renin with a reduced bond (—CH₂—NH—). These compounds also have shown a high degree of species specificity. Boger and colleagues tried another approach, incorporating statine into peptide analogues of angiotensinogen. Unfortunately, none of these substrate analogues are orally active.

In our preliminary report, we synthesized small peptides in the ANG I sequence, with leucinal at the C terminus, and showed that benzyloxycarbonyl("Z")-Phe-His-leucinal and its derivatives were potent inhibitors of human renin in vitro. In this study we report in vitro experiments with derivatives of Z-Phe-His-leucinal and with new small peptide analogues containing statine that are highly potent and species-specific inhibitors of human renin.

Materials and Methods

DE-52 was purchased from Whatman, Ltd., Maidstone, Kent, U.K. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. ¹²⁵I-ANG I was from New England Nuclear, Boston, Massachusetts. ANG I, ANG II, pepstatin A, and hippurylhistidylleucine (Hip-His-Leu) were from Protein Research Foundation, Mino, Osaka, Japan. Homogeneous human kidney ACE used in this study was previously described. ¹⁵

Human kidney renin, prepared by the method described previously, contained 0.7 Goldblatt units/mg.
of protein when calibrated with standard human renin. Monkey, pig, goat, dog, rabbit, and rat renins, which were prepared by the method described previously, were further purified by gel filtration on Ultrogel AcA 44 (LKB, Bromma, Sweden).

Human angiotensinogen was prepared from pooled plasma by ammonium sulfate precipitation (between 1.5 and 2.3 M), anti-human angiotensinogen antibody-Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) affinity column chromatography, and DEAESephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography (unpublished data). The preparation contained 7.5  μg of ANG I equivalents/mg of protein. Sheep, rabbit, and rat angiotensinogens from bilaterally nephrectomized animals were prepared according to the method of Sen et al.

The test compounds were dissolved in 60% ethyl alcohol. Human renin activity in the presence and absence of each compound was measured using sheep angiotensinogen or human angiotensinogen. Monkey renin activity was measured with sheep angiotensinogen, and the homologous angiotensinogens were used with rabbit and rat renins. Pig, goat, and dog renin activities were measured with hog angiotensinogen (2275 units/mg of protein; Sigma Chemical Co., St. Louis, MO). The total 1-ml assay mixture contained 0.1 M phosphate buffer, pH 7.3, human renin (0.5 ng of ANG 1/minute), sheep or human angiotensinogen (200 ng of ANG I equivalents), seven different concentrations of each compound, 6% ethyl alcohol, and angiotensinase inhibitors (10 mM of EDTA and 3.4 mM of 8-hydroxyquinoline). After 10 minutes at 37°C, the reaction was stopped by placing the tubes in a boiling water bath for 5 minutes. After centrifugation, the supernatant (50–100 μL) was used for assay of ANG I. The generated ANG I was measured by radioimmunoassay. Activities of the animal renins and total amounts of ANG I released from the respective angiotensinogens in the incubation mixtures were the same as those of human renin and angiotensinogen as described above.

The Kₜ values for each compound were determined by the Dixon plot for two different substrate concentrations (100 ng and 200 ng of ANG I equivalents) in the incubation mixtures already described. The reaction was conducted for 10 minutes at 37°C.

Cathepsin D (bovine spleen; 12.5 units/mg of protein; Sigma Chemical Co., St. Louis, MO) activity was measured using bovine hemoglobin (Sigma) as substrate by the method of Barrett. Pepsin (porcine stomach; 2965 units/mg of protein; Sigma) was assayed using bovine hemoglobin as substrate by the method of Ryle. The ACE activity was measured using Hip-His-Leu as substrate by the method described previously. Protein was measured by the method of Lowry et al. with bovine serum albumin as the standard.

**Results**

None of the compounds tested cross-reacted with ANG I antibody at the concentration of 10⁻⁴ M. Pepstatin A was used as a standard renin inhibitor in the human renin and sheep angiotensinogen reaction system. Pepstatin A inhibited human renin with an IC₅₀ (inhibitory concentration, 50%) of 2.0 × 10⁻⁶ M.

The renin inhibitory potencies of small peptide analogues and their derivatives are shown in Table 1. The

| Table 1 Structures of Synthetic Peptide Analogues and Their Inhibitory Potencies Against Human Renin |
|---|---|---|
| Code number | Compound | IC₅₀ (M) sheep angiotensinogen | Kₜ (M) human angiotensinogen |
| ES-1089 | Z-Tyr-Ile-His-Pro-Phe-His-leucinal* | No inhibition | |
| ES-1090 | Z-Ile-His-Pro-Phe-His-leucinal* | No inhibition | |
| ES-1091 | Z-His-Pro-Phe-His-leucinal* | 5.0 × 10⁻⁵ | |
| ES-1092 | Z-Pro-Phe-His-leucinal* | 3.2 × 10⁻⁷ | 1.1 × 10⁻⁷† |
| ES-1093 | Z-Phe-His-leucinal* | 7.5 × 10⁻⁷ | |
| ES-181 | Z-Phe-His-leucinol | > 10⁻⁴ | |
| ES-1094 | Z-His-leucinal* | 5.0 × 10⁻⁴ | |
| ES-188 | Z-[3-(1-naphthyl)Ala]-His-leucinal* | 8.0 × 10⁻⁸ | 2.4 × 10⁻⁷ |
| ES-212 | Z-[3-(1-naphthyl)Ala]-His-leucinol* | 5.6 × 10⁻⁶ | 3.5 × 10⁻⁶ |
| ES-215 | Z-[3-(2-naphthyl)Ala]-His-leucinol | > 10⁻⁴ | |
| ES-241 | Ac-d,L-[3-(9-phenanthryl)Ala]-His-leucinol | > 10⁻⁴ | |
| ES-242 | Ac-d,L-[3-(9-anthraceny)Ala]-His-leucinol | No inhibition | |
| ES-216 | Z-Phe-His-statine | > 10⁻⁴ | |
| ES-217 | Z-[3-(1-naphthyl)Ala]-His-statine | 6.4 × 10⁻⁶ | |
| ES-226 | Z-[3-(1-naphthyl)Ala]-His-statine ethyl ester | 2.8 × 10⁻⁷ | 2.3 × 10⁻⁷ |
| ES-254 | Z-[3-(1-naphthyl)Ala]-His-statine 2-methylbutylamide | 4.5 × 10⁻⁷ | 1.9 × 10⁻⁷ |

Each value is the mean of three or four determinations

Z = benzyloxycarbonyl, Ac = acetyl; IC₅₀ = inhibitory concentration, 50%

*Ref 14

†Sheep angiotensinogen was used as substrate
The minimal sequence required to inhibit human renin was the tripeptide aldehyde Z-Phe-His-leucinal, whereas the hexapeptide aldehyde Z-Ile-His-Pro-Phe-His-leucinal had no inhibitory effect on human renin at the concentration of $5 \times 10^{-4}$ M. When the aldehyde group of leucinal at the C terminus of compounds ES-1093 or ES-188 was substituted with an alcohol group, as in ES-181 or ES-212, the inhibitory potency was markedly reduced. On the other hand, replacement of the benzene ring of phenylalanine with 1-naphthalene increased the inhibitory potency tenfold. Replacement of the benzene ring with 2-naphthalene, 9-phenanthrene or 9-anthracene caused more than a 1000-fold decrease in the inhibitory potency. Replacing leucine residue at the C terminus of ES-188 with statine caused no substantial improvement in the inhibitory potency.

The $K_i$ values of ES-188, ES-226, and ES-254 for human renin with human angiotensinogen were similar ($10^{-7}$ M). The inhibition of ES-188 was noncompetitive, whereas those of ES-226 and ES-254 were competitive. When the aldehyde group of leucinal at the C terminus of ES-188 was substituted with an alcohol group, inhibition changed from noncompetitive to competitive.

The inhibitory effects of ES-188, ES-226, and ES-254 on six different species of animal renins were studied (Table 2). These three inhibitors demonstrated similar potency in inhibiting monkey and human renin but were about one or two orders of magnitude less active against rabbit renin. They were very weak inhibitors of pig, goat, dog, and rat renins.

We tested the abilities of compounds ES-188, ES-226 and ES-254 to inhibit aspartyl proteases, such as cathepsin D and pepsin, and human kidney ACE. ES-188 had no discernible effect on cathepsin D, pepsin, or ACE at the concentration of $10^{-4}$ M. Compound ES-254, a statine-containing analogue, had some inhibitory effect on cathepsin D ($IC_{50}$ of $1.4 \times 10^{-5}$ M) and pepsin ($IC_{50}$ of $4.2 \times 10^{-5}$ M). It inhibited human kidney ACE with an $IC_{50}$ of $7.1 \times 10^{-5}$ M. Another statine-containing analogue, compound ES-226, had little inhibitory effect on cathepsin D, pepsin, or ACE at the concentration of $10^{-3}$ M.

### Discussion

Development of orally effective inhibitors of renin is desirable because the ACE inhibitors being used in experimental and clinical studies are not physiologically specific inhibitors of the renin-angiotensin system. A specific inhibitor of renin that could be used in clinical studies would help define the role of the renin-angiotensin system in essential hypertension.

The in vitro results presented here demonstrate the high potency against human and primate renins of small peptide analogues that have aldehydic or statine-containing residues at the C terminus. Several peptidyl amino aldehydes of microbial origin, such as leupeptin, antipain, and chymostatin, are potent inhibitors of serine protease or thiol protease. We postulated that small peptides in ANG I sequence with leucinal at the C terminus might be precursors of transition state analogues. Small peptide aldehydes that inhibited human renin were simultaneously reported by us and by Boger et al. Tripeptide aldehydes synthesized by Boger et al. were more than tenfold less potent than the Z-Phe-His-leucinal we reported. To improve active site binding affinity of small peptide analogues, we replaced the benzene ring of phenylalanine with 1-naphthalene, 2-naphthalene, 9-phenanthrene, or 9-anthracene. Only 1-naphthylalanine-containing peptide analogues showed a high potency in inhibiting human renin. These results suggest that 1-naphthylalanine fits more closely than phenylalanine in the pocket (Subsite S3) of the active site cleft of human renin.

Boger et al. have reported statine-containing renin inhibitors that are highly potent against human renin and canine renin. Their compounds retained a relatively high potency against pig and rat renins and had longer amino acid sequences than the statine-containing inhibitors used in this study. Our compounds showed a very weak potency against pig, goat, dog, and rat renins. These findings of species differences may be related to differences in the three-dimensional structure of the substrate binding site of the respective renins.

Renin is a carboxyl protease and has a three-dimensional structure similar to that of other carboxyl proteases. This suggests that a renin inhibitory compound may have an inhibitory potency against other carboxyl proteases. Statine-containing compounds synthesized by Boger et al., by Tree et al., and by us had a considerable degree of inhibitory effect on pepsin and cathepsin D. However, orally active renin inhibitors should not inhibit pepsin.

In conclusion, our results indicate that 1-naphthylalanine-containing short peptide analogues are highly potent human renin inhibitors. This family of inhibitors eventually may yield an orally active renin inhibitor for the treatment of hypertension.

### Table 2. Inhibitory Effects of ES-188, ES-226, and ES-254 on Six Animal Renins

<table>
<thead>
<tr>
<th>Renin</th>
<th>Angiotensinogen</th>
<th>ES-188</th>
<th>ES-226</th>
<th>ES-254</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey</td>
<td>Sheep</td>
<td>52</td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Rabbit</td>
<td>70</td>
<td>300</td>
<td>750</td>
</tr>
<tr>
<td>Rat</td>
<td>Rat</td>
<td>2900</td>
<td>3000</td>
<td>33%</td>
</tr>
<tr>
<td>Dog</td>
<td>Hog</td>
<td>3400</td>
<td>38%</td>
<td>35%</td>
</tr>
<tr>
<td>Hog</td>
<td>Hog</td>
<td>37%</td>
<td>24%</td>
<td>21%</td>
</tr>
<tr>
<td>Goat</td>
<td>Hog</td>
<td>23%</td>
<td>20%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Values are the mean of three or four measurements. Percent indicates the inhibitory percent of each inhibitor at the concentration of $10^{-4}$ M.

$IC_{50}$ = inhibitory concentration, 50%.
References


Highly potent and specific inhibitors of human renin.
T Kokubu, K Hiwada, E Murakami, Y Imamura, R Matsueda, Y Yabe, H Koike and Y Iijima

Hypertension. 1985;7:I8
doi: 10.1161/01.HYP.7.3_Pt_2.I8

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/7/3_Pt_2/I8

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/