Highly Potent and Specific Inhibitors of Human Renin

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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 x 10^-5 M), pepsin (IC50 of 4.2 x 10^-5 M), and human angiotensin-converting enzyme (IC50 of 7.1 x 10^-6 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 inhibitors1-2 and the clear demonstration of their efficacy as antihypertensive drugs in patients with renin-dependent hypertension and essential hypertension3-5 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.6 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,7-10 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—).11 These compounds also have shown a high degree of species specificity.12 Boger and colleagues13 tried another approach, incorporating statine into peptide analogues of angiotensinogen. Unfortunately, none of these substrate analogues are orally active.

In our preliminary report,14 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.121-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, Minoh, Osaka, Japan. Homogeneous human kidney ACE used in this study was previously described.15

Human kidney renin, prepared by the method described previously,16 contained 0.7 Goldblatt units/mg

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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 DEAE-Sephalac (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 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 radioimmunossay. 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 × 10⁻⁶ M.

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

<table>
<thead>
<tr>
<th>Code number</th>
<th>Compound</th>
<th>IC₅₀ (M) sheep angiotensinogen</th>
<th>IC₅₀ (M) human angiotensinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES-1089</td>
<td>Z-Tyr-Ile-His-Pro-Phe-His-leucinal*</td>
<td>No inhibition</td>
<td></td>
</tr>
<tr>
<td>ES-1090</td>
<td>Z-Ile-His-Pro-Phe-His-leucinal*</td>
<td>No inhibition</td>
<td></td>
</tr>
<tr>
<td>ES-1091</td>
<td>Z-His-Pro-Phe-His-leucinal*</td>
<td>5.0 × 10⁻⁵</td>
<td></td>
</tr>
<tr>
<td>ES-1092</td>
<td>Z-Pro-Phe-His-leucinal*</td>
<td>3.2 × 10⁻⁷</td>
<td>1.1 × 10⁻⁷†</td>
</tr>
<tr>
<td>ES-1093</td>
<td>Z-Phe-His-leucinal*</td>
<td>7.5 × 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>ES-181</td>
<td>Z-Phe-His-leucinol</td>
<td>&gt; 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>ES-1094</td>
<td>Z-His-leucinal*</td>
<td>5.0 × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>ES-188</td>
<td>Z-[3-(1-naphthyl)Ala]-His-leucinal*</td>
<td>8.0 × 10⁻⁸</td>
<td>2.4 × 10⁻⁷</td>
</tr>
<tr>
<td>ES-212</td>
<td>Z-[3-(1-naphthyl)Ala]-His-leucinol*</td>
<td>5.6 × 10⁻⁶</td>
<td>3.5 × 10⁻⁶</td>
</tr>
<tr>
<td>ES-215</td>
<td>Z-[3-(2-naphthyl)Ala]-His-leucinol</td>
<td>&gt; 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>ES-241</td>
<td>Ac-d-l-[3-(9-phenanthyl)Ala]-His-leucinal</td>
<td>&gt; 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>ES-242</td>
<td>Ac-d-l-[3-(9-anthracenyl)Ala]-His-leucinol</td>
<td>No inhibition</td>
<td></td>
</tr>
<tr>
<td>ES-216</td>
<td>Z-Phe-His-statineol</td>
<td>&gt; 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>ES-217</td>
<td>Z-[3-(1-naphthyl)Ala]-His-statinol</td>
<td>6.4 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>ES-226</td>
<td>Z-[3-(1-naphthyl)Ala]-His-statine ethyl ester</td>
<td>2.8 × 10⁻⁷</td>
<td>2.3 × 10⁻⁷</td>
</tr>
<tr>
<td>ES-254</td>
<td>Z-[3-(1-naphthyl)Ala]-His-statine 2-methylbutyramide</td>
<td>4.5 × 10⁻⁷</td>
<td>1.9 × 10⁻⁷</td>
</tr>
</tbody>
</table>

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
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 $5 \times 10^{-4} \text{ M}$. 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-phenanth-
rene 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
caus ed 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
($10^{-2} \text{ M}$). The inhibition of ES-188 was noncompeti-
tive, whereas those of ES-226 and ES-254 were com-
petitive. When the aldehyde group of leucinal at the C
terminus of ES-188 was substituted with an alcohol
group, inhibition changed from noncompetitive to com-
petitive.

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 in-
hibitors 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 $10^{-4} \text{ M}$. Compound
ES-254, a statine-containing analogue, had some inhibi-
tory effect on cathepsin D ($IC_{50}$ of $1.4 \times 10^{-3} \text{ M}$)
and pepsin ($IC_{50}$ of $4.2 \times 10^{-3} \text{ M}$). It inhibited human
kidney ACE with an $IC_{50}$ of $7.1 \times 10^{-4} \text{ M}$. Another
statine-containing analogue, compound ES-226, had
little inhibitory effect on cathepsin D, pepsin, or ACE
at the concentration of $10^{-3} \text{ M}$.

### Discussion

Development of orally effective inhibitors of renin
is desirable because the ACE inhibitors being used in
experimental and clinical studies are not physiologi-
cally specific inhibitors of the renin-angiotensin sys-
tem. 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 leupep-
tin, 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 ana-
logues. Small peptide aldehydes that inhibited human
renin were simultaneously reported by us and by Feh-
rentz et al. Tripeptide aldehydes synthesized by Feh-
rentz 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 pep-
tide analogues showed a high potency in inhibiting
human renin. These results suggest that 1-naphthyl-
alanine fits more closely than phenylalanine in the
 pocket (Subsite $S_3$) 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-contain-
ing 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-dimen-
sional structure similar to that of other carboxyl pro-
teases. This suggests that a renin inhibitory com-
 pound 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 pep-
sin and cathepsin D. However, orally active renin
inhibitors should not inhibit pepsin.

In conclusion, our results indicate that 1-naphtyl-
alanine-containing short peptide analogues are highly
potent human renin inhibitors. This family of inhibi-
tors eventually may yield an orally active renin inhibi-
tor 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>IC$_{50}$ (x $10^{-4}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey</td>
<td>Sheep</td>
<td>5.2</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Rabbit</td>
<td>7.0</td>
</tr>
<tr>
<td>Rat</td>
<td>Rat</td>
<td>2900</td>
</tr>
<tr>
<td>Dog</td>
<td>Hog</td>
<td>3400</td>
</tr>
<tr>
<td>Hog</td>
<td>Hog</td>
<td>37%</td>
</tr>
<tr>
<td>Goat</td>
<td>Goat</td>
<td>23%</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 $10^{-4} \text{ M}$.

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


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

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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