Statine-Containing Dipeptide and Tripeptide Inhibitors of Human Renin

TATSUO KOKUBU, KUNIO HIWADA, AKIHIRO NAGAE, EIKI MURAKAMI, YASUHIRO MORISAWA, YUICHIRO YABE, HIROYUKI KOIKE, AND YASUTERU IJIMA

SUMMARY Dipeptide and tripeptide derivatives containing a statine residue were synthesized as inhibitors of human renin. ES-305, bis[(1-naphthyl)methyl]acetyl(BNMA)-histidyl-statine 2(S)-methylbutylamide was found to be a highly potent inhibitor of human renin with a $K_i$ value of $1.7 \times 10^{-8}$ M. Dipeptide derivatives with the BNMA group at the N-terminal (BNMA-VaJ-Sta-isoleucinol [ES-313], BNMA-Leu-Sta-isoleucinol [ES-316], and BNMA-Nle-Sta-isoleucinol [ES-317]) had potencies against human renin that were similar to the potency of ES-305. All these dipeptide derivatives competitively inhibited human renin. The inhibitors were also potent against monkey renin but were less effective against renins from pig, goat, dog, rabbit, and rat. ES-305 had little effect on cathepsin D and pepsin at the concentration of $10^{-7}$ M. The other derivatives showed detectable inhibition of cathepsin D ($IC_{50}=10^{-6}$-10$^{-7}$ M) and pepsin ($10^{-5}$-10$^{-4}$ M). All the compounds had little or no effect on trypsin, chymotrypsin, angiotensin converting enzyme, and urinary kallikrein at the concentration of $10^{-7}$ M. Our results indicate that ES-305 is a highly potent and specific inhibitor of human renin. This compound is superior to other, previously described statine-containing renin inhibitors with respect to molecular size and enzyme specificity. (Hypertension 8 [Suppl II]: II-1-II-5, 1986)

Key Words • renin inhibitors • renal renin • plasma renin • cathepsin D • pepsin • bis[(1-naphthyl)methyl]acetyl group

RENIN (EC 3.4.23.15) selectively cleaves its protein substrate, angiotensinogen, to release the decapeptide angiotensin I, which in turn is converted to the potent pressor octapeptide angiotensin II by angiotensin converting enzyme (ACE; EC 3.4.15.1). ACE inhibitors have been shown to decrease the formation of angiotensin II in vivo and are now widely used in the treatment of hypertension and congestive heart failure. The development of orally effective renin inhibitors may provide an alternative way of blocking the renin-angiotensin system.

In previous papers, we reported the synthesis of small peptide analogues representing the C-terminal portion of the angiotensin I sequence. Among them, benzyloxy carbonyl (Z)-3-(1-naphthyl)alanine-containing tripeptide analogues were highly potent inhibitors of human renin with inhibitor constant values in the range of $10^{-7}$ to $10^{-8}$ M. The research groups of Boger et al. and Tree et al. have synthesized highly potent inhibitors of human renin by incorporating a statine residue into peptide analogues of angiotensinogen. Our statine-containing peptide analogues ES-226 [Z-3-(1-naphthyl)Ala-His-statine ethyl ester] and ES-254 [Z-3-(1-naphthyl)Ala-His-statine 2(S)-methylbutylamide] were smaller but were about one or two orders of magnitude less potent than those reported by the two other research groups. None of the compounds, including ours, are orally active.

As part of an effort to develop orally active and renin-specific inhibitors, we have synthesized additional statine-containing peptide derivatives. This paper reports in vitro experiments with statine-containing dipeptide and tripeptide derivatives that are highly potent inhibitors of human renin.

Materials and Methods

125I-labeled angiotensin I was purchased from New England Nuclear (Boston, MA, USA). Angiotensin I, pepstatin A, hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and N-benzoyl-L-arginine 4-nitroanilide HCl (Bz-Arg-p-NA) were obtained from the Peptide Insti...
tute (Minoh, Osaka, Japan). N-Succinyl-L-phenylalanine 4-nitroanilide (Suc-Phe-p-NA) and hog angiotensinogen (1.7 U/mg of protein) were provided by Sigma (St. Louis, MO, USA). D-Valyl-L-leucyl-L-arginine 4-nitroanilide 2HCl (D-Val-Leu-Arg-p-NA) was obtained from Kabi Diagnostica (Stockholm, Sweden).

Human kidney renin, prepared by a method described previously, contained 0.84 Goldblatt units/mg of protein when calibrated in vitro with standard human renin. Monkey (Macaca irius), pig (Middle Yorkshire), goat (Saanaen), dog (mongrel), rabbit (Japanese White), and rat (Wistar) renins used in this study have been described elsewhere.

Human angiotensinogen was prepared from pooled plasma by a method described previously. The preparation contained 6.4 µg of angiotensin I equivalents per milligram of protein. Sheep (Corriedale), rabbit (Japanese White), and rat (Wistar) angiotensinogens used in this study have been described elsewhere.

Human plasma with high plasma renin activity (more than 5 ng of angiotensin I per milliliter per hour) was collected from patients with various disorders, excluding any patients receiving treatment with ACE inhibitor, and was pooled.

Homogeneous human kidney ACE used in this study has been described previously. Human urinary kallikrein was partially purified by DE-52 (Whatman, Maidstone, Kent, UK) column chromatography (unpublished data). The preparation contained 4.5 kallikrein units (KU) per milligram of protein.

The test compounds were dissolved in 60% ethyl alcohol. Human renin activity in the presence and absence of each compound was measured with human angiotensinogen. The total 1-ml assay mixture contained 0.05 M phosphate buffer (pH 7.3), human renin (about 0.5 ng of angiotensin I per minute), seven different concentrations of each compound, 6% ethyl alcohol, and angiotensinase inhibitors (10 mM ethylenediaminetetraacetic acid (EDTA) and 3.4 mM 8-hydroxyquinoline). The incubation was carried out at 37°C for 2 hours, and the reaction was stopped by placing the tubes in a boiling water bath for 5 minutes. After centrifugation, the amount of angiotensin I generated in the supernatant (50–100 µl) was measured by radioimmunoassay.

The inhibitor constant (K) values for each compound were determined by the Dixon plot for two different angiotensinogen concentrations (100-ng and 200-ng angiotensin I equivalents) in the assay medium already described. The reaction was performed at 37°C for 10 minutes.

Cathepsin D (bovine spleen; 13 U/mg of protein; Sigma) was measured in the presence and absence of each compound, with bovine hemoglobin (Sigma) used as substrate, by the method of Barrett. Pepsin (porcine stomach; 3630 U/mg of protein; Sigma) was assayed with bovine hemoglobin as substrate, by the method of Ryle. Trypsin (bovine pancreas, type I; 10,500 BAEE U/mg of protein; Sigma) activity was measured with Bz-Arg-p-NA as substrate, by the method of Geiger and Fritz. Chymotrypsin (bovine pancreas; 43 U/mg of protein; Sigma) was assayed with Suc-Phe-p-NA as substrate, by the method of Geiger. ACE activity was measured with Hip-His-Leu as substrate, by a method we have described previously. The activity of urinary kallikrein was measured with D-Val-Leu-Arg-p-NA as substrate, by the colorimetric method. 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 angiotensin I antibody at the concentration of 10^{-4} M. Pepstatin A, which was used as a standard renin inhibitor in the human renin and human angiotensinogen reaction, inhibited human renin, with a 50% inhibitory concentration (IC_{50}) of 3.2 × 10^{-5} M at pH 7.3.

The chemical structures of dipeptide and tripeptide derivatives and their inhibitory potencies against human renal and plasma renin are shown, respectively, in Figure 1 and Table 1. All the compounds containing a statine residue inhibited human renin, with IC_{50} values in the range of 10^{-8} M. The dipeptide derivatives and one of the tripeptide derivatives (ES-309) also inhibited human plasma renin activity by 50% at similar concentrations (Table 1).

The replacement of the benzoyloxycarbonyl-3-(1-naphthyl)alanine group of ES-254 [Z-3-(1-naphthyl)Ala-His-statine 2(S)-methylbutylamide] with the bis[(1-naphthyl)methyl]acetyl group (ES-305), as shown in Figure 1, increased the inhibitory potency 100-fold. The dipeptide derivatives in which the histidine residue of ES-305 was replaced with valine (ES-313), leucine (ES-316), or norleucine (ES-317) were found to be essentially as potent as the compound ES-305. The K_{i} values of these compounds for human renin with human angiotensinogen were similar (10^{-9} M). These statine-containing dipeptide and tripeptide derivatives competitively inhibited human renin. Rep-
FIGURE 1. Chemical structures of seven statine-containing dipeptide and tripeptide derivatives.

Representative Dixon plots of ES-305 and ES-317 are shown in Figure 2.

The inhibitory effects of the seven compounds were studied in renins from six different species of animals (Table 2). The human renin inhibitors were similarly potent in inhibiting monkey renin but were about one to four orders of magnitude less active against renins from rabbit, dog, goat, and pig. The compounds ES-305, ES-309, and ES-313 were very weak inhibitors of rat renin.

The inhibitory effects of seven human renin inhibitors on aspartyl proteases, such as cathepsin D and pepsin, were also studied. ES-305 had a very weak

![Figure 2. Dixon plots of the inhibition of human renin by ES-305 and ES-317 for two concentrations of human angiotensinogen (100 and 200 ng of angiotensin I equivalents per milliliter of assay volume). Human renin activity was 0.5 ng of angiotensin I per milliliter of assay volume per minute at pH 7.3 and 37°C. The reaction was carried out for 10 minutes at pH 7.3 and 37°C under the conditions described in the text. ES-305 = bis[(1-naphthyl)acetyl]-histidyl-statine 2(S)-methylbutylamide. ES-317 = bis[(1-naphthyl)acetyl]-norleucyl-statyl-isoleucinol.

Table 1. Inhibitory Potencies of Seven Statine-Containing Dipeptide and Tripeptide Derivatives Against Human Renin

<table>
<thead>
<tr>
<th>Code number</th>
<th>Compound</th>
<th>Human renal renin-human angiotensinogen</th>
<th>Human plasma renin-endogenous angiotensinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (M)</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; (M)</td>
</tr>
<tr>
<td>ES-295</td>
<td>Z-3-(1-naphthyl)Ala-His-Sta-isoleucine-4-amino-1-benzylpiperidine</td>
<td>4.0 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>3.7 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>ES-300</td>
<td>2-(2-methoxyethoxy)ethoxy-carbonylalanine-alanyl-His-statine 2(S)-methylbutylamide</td>
<td>8.2 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>6.5 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>ES-305</td>
<td>N&lt;sup&gt;γ&lt;/sup&gt;-BNMA-His-statine 2(S)-methylbutylamide</td>
<td>2.4 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.7 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>ES-309</td>
<td>Z-3-(1-naphthyl)Ala-Leu-Sta-isoleucinol</td>
<td>2.5 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>ES-313</td>
<td>BNMA-Val-Sta-isoleucinol</td>
<td>3.6 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>2.5 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>ES-316</td>
<td>BNMA-Leu-Sta-isoleucinol</td>
<td>4.8 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>3.0 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>ES-317</td>
<td>BNMA-Nle-Sta-isoleucinol</td>
<td>1.5 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.2 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each value is the mean of three determinations. IC<sub>50</sub> = inhibitory concentration, 50%; K<sub>i</sub> = inhibitor constant; Z = benzyloxycarbonyl; BNMA = bis[(1-naphthyl)acetyl]; Nle = norleucine.
potency in inhibiting cathepsin D and pepsin, although it contains a statine residue. The other dipeptide and tripeptide derivatives showed detectable inhibition of cathepsin D and pepsin. The compounds tested had little or no inhibitory effects on human kidney ACE, human urinary kallikrein, bovine trypsin, and bovine chymotrypsin, except for ES-295, which inhibited human ACE (IC$_{50}$, $2.8 \times 10^{-6}$ M) at a concentration of $10^{-2}$ M (Table 3).

**Discussion**

The success of ACE inhibitors in the treatment of patients with hypertension and congestive heart failure has stimulated the development of inhibitors specific for human renin. Renin is known to have only one substrate, whereas ACE has several substrates other than angiotensin I. Several academic and industrial groups, including ours, have synthesized many compounds in search of specific renin inhibitors. Orally active renin inhibitors are not available at present.

The in vitro results presented here indicate that the statine-containing dipeptide and tripeptide analogues have a high potency against human renin as well as primate renin. In our previous paper, we showed that Z-3-(1-naphthyl)alanine-containing tripeptide analogues were highly potent inhibitors of human renin. Unfortunately, however, they were not orally effective. To enhance their potency, we tried to increase the tightness of binding of the inhibitors to the active site of human renin. The replacement of the Z-3-(1-naphthyl)alanine group at the N-terminal of ES-254 with the bis[(1-naphthyl)methyl]acetyl group resulted in a 100-fold increase in potency. The renin inhibitory potencies of these compounds containing the bis[(1-naphthyl)methyl]acetyl group are equal to the potencies of human renin inhibitors reported by Boger et al. Recently, Székely and his co-workers reported a powerful human renin inhibitor (H-261 [Boc-His-Pro-Phe-His-Leu(OH)-Val-Ile-His]; IC$_{50}$, $6.9 \times 10^{-10}$ M) containing the hydroxy isostere at the scissile bond. H-261, however, is composed of eight amino acid residues. So far as we know, ES-305 is the shortest peptide inhibitor of human renin reported in the world literature.

The synthetic compounds with statine or statinol at the C-terminal were found to be weak inhibitors of human renin. The presence of a protected statine moiety, such as statine ethyl ester or statine ethyl ester containing the bis[(1-naphthyl)alanine group at the M-terminal of ES-254, is important for a powerful human renin inhibitor. The replacement of the Z-3-(1-naphthyl)alanine group at the N-terminal of ES-254 with the bis[(1-naphthyl)methyl]acetyl group resulted in a 100-fold increase in potency. The renin inhibitory potencies of these compounds containing the bis[(1-naphthyl)methyl]acetyl group are equal to the potencies of human renin inhibitors reported by Boger et al. Recently, Székely and his co-workers reported a powerful human renin inhibitor (H-261 [Boc-His-Pro-Phe-His-Leu(OH)-Val-Ile-His]; IC$_{50}$, $6.9 \times 10^{-10}$ M) containing the hydroxy isostere at the scissile bond. H-261, however, is composed of eight amino acid residues. So far as we know, ES-305 is the shortest peptide inhibitor of human renin reported in the world literature.

**Table 3. Effects of Seven Statine-Containing Dipeptide and Tripeptide Derivatives on Six Different Kinds of Proteases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitory percent at the concentration of $10^{-3}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin D (bovine)</td>
<td>Hemoglobin</td>
<td>66.8 (3.9) 28.1 (42) 4.7 40.9 (11) 97.1 (0.84) 65.7 (3.6) 65.7 (3.6)</td>
</tr>
<tr>
<td>Pepsin (porcine)</td>
<td>Hemoglobin</td>
<td>78.2 (2.4) 12.6 7.8 83.8 (0.82) 82.8 (1.5) 55.5 (17) 55.5 (17)</td>
</tr>
<tr>
<td>Trypsin (bovine)</td>
<td>Bz-Arg-p-NA</td>
<td>6.9 6.2 7.5 2.5 0 10 10</td>
</tr>
<tr>
<td>Chymotrypsin (bovine)</td>
<td>Suc-Phe-p-NA</td>
<td>0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>ACE (human)</td>
<td>Hip-His-Leu</td>
<td>84.2 (2.8) 0 24.8 0 6.0 4.0 3.0</td>
</tr>
<tr>
<td>Urinary kallikrein (human)</td>
<td>d-Val-Leu-Arg-p-NA</td>
<td>0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

Each value is the mean of three measurements. The value in parentheses indicates IC$_{50}$ ($\times 10^{-6}$ M). IC$_{50}$ = inhibitory concentration, 50%; ACE = angiotensin converting enzyme.
effects of the statine-containing peptide analogues on cathepsin D and pepsin, without losing their strong inhibitory effects on human renin. Orally active renin inhibitors used for the treatment of patients should not inhibit pepsin.

Recently, the three-dimensional structure of human renin has been modeled after that of other aspartyl proteases.\(^{24-26}\) Examination of the model suggests that the changes in the amino acids in the active site could explain the differences in species specificity.\(^{24-26}\) Our present and previous human renin inhibitors\(^{6-8}\) have a similar inhibitory effect on monkey renin, suggesting a similarity in its active site. As discussed by Carlson et al.,\(^{26}\) the subtle changes in the amino acids that line the active site cleft could account for the decrease in the potency of our compounds in inhibiting other animal renins tested. The compound ES-305 may offer a probe for modeling the structure of the active site of human renin more precisely, thus providing further insight into the mechanism of action of renin and also into the design of additional powerful inhibitors.

In conclusion, our results demonstrate that bis[(1-naphthyl)methyl]acetyl-histidyl-statine 2(S)-methylbutyramide (ES-305) is a highly potent and specific inhibitor of human renin. This new inhibitor is more potent than our earlier inhibitors. The other dipeptide derivatives with the bis[(1-naphthyl)methyl]acetyl group have potencies against human renin that are similar to the potency of ES-305, but they also have some inhibitory effects on cathepsin D and pepsin. ES-305 may eventually yield an orally effective renin inhibitor.

References

22. Haber E. Which inhibitors will give us true insight into what renin really does? J Hypertension 1984;2:223-230
Statine-containing dipeptide and tripeptide inhibitors of human renin.
T Kokubu, K Hiwada, A Nagae, E Murakami, Y Morisawa, Y Yabe, H Koike and Y Iijima

Hypertension. 1986;8:II1
doi: 10.1161/01.HYP.8.6_Pt_2.II1

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/8/6_Pt_2/II1