NEW RENIN INHIBITORS – STABILITY AND ACTIVITY DETERMINATION. PART III

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Abstract: A series of new four potential renin inhibitors containing pseudodipeptides were synthesized. Stability for all compounds (1-4) in homogenates of liver, kidney, lung and in serum, gastric, intestinal juice and in the presence of α-chymotrypsin was determined. Compound 1 was unstable, compounds 2, 3 were stable, compound 4 was partly unstable, (liver and kidney homogenates, α-chymotrypsin solution). Inhibitory activity of the compounds was measured in vitro by HPLC determination of lowering concentration of substrate (angiotensinogen) in the presence of renin and the potential renin inhibitor (compounds 1-4). Compound 1, 2, 3 and 4 showed inhibitory activity (1.7 × 10⁻⁶, 9.6 × 10⁻⁷, 1.05 × 10⁻⁹ and 1.31 × 10⁻⁷ M, respectively).

Keywords: HPLC, activity of potential renin inhibitors, renin inhibitors

Hypertension is a major risk factor for cardiovascular disease, including chronic heart and kidney failures, myocardial infarction and stroke. Improvements in the therapy are necessary in order to decrease the morbidity and mortality associated with hypertension and its associated diseases.

There are a variety of medications available to treat hypertension, such as agents which block the renin-angiotensin-aldosterone system, angiotensin converting enzyme inhibitors and ANGII receptor blockers, but still many patients with hypertension, using hypertensive medicines do not have their blood pressure controlled to recommended target levels (< 140/90) (1, 2). Searching for the new antihypertensive agents has led to rapid development of potent renin inhibitors. Suppression of the renin-angiotensin system, which plays an important role in regulation of blood pressure and fluid homeostasis, by inhibition of renin activity may be an effective way for antihypertensive therapy (3, 4). Therefore the new active renin inhibitors are developed. In the search of new renin inhibitors with sufficient inhibiting activity many new peptide analogs were synthesized (3, 5-13). At present, studies on the design and synthesis of novel renin inhibitors are focused on developing nonpeptides, containing heterocyclic rings e.g., piperidine (4, 14), piperazine (15), isoxazole (16), pyrimidine (14, 17). Aliskiren is the first nonpeptide, small-molecule renin inhibitor for the treatment of hypertension. The studies suggest that aliskiren is at least as good as existing agents for blood pressure regulation in patients with mild to moderate hypertension (2, 18-21). Searching for new pseudopeptide renin inhibitors ditekiren was synthesized, the highly potent inhibitor of human renin (22).

In two recent reports (1, 23) we described the determination of enzymatic stability and inhibiting activity of 12 newly synthesized potential renin inhibitors containing pseudopetides. Our intention was to check if the new compounds were resistant to enzymatic degradation, metabolically stable and to determine the inhibitory activity.

and made compound more stable. Compound with AHBA group showed activity 10⁻⁴M and was rather stable (decomposed only in kidney homogenate). Compound 4 had four times lower activity than compound 2 and was rather stable (decomposed only in liver homogenate), the half-time was about 60 min. The presence of triethyl group caused that compound was unstable in all homogenates and body fluids. Removal of Boc group increased metabolic stability and increased solubility in water (23).

In Part II of our previous paper we described other six new synthesized renin inhibitors: Boc-Phe-(4-OMe)-His(N^Bzl)-AHNA-OEt (1), Boc-Phe-(4-OMe)-His(N^Bzl)-AHNA-εAhx-Iaa (2), Boc-Phe-(4-OMe)His(N^trit)-AHNA-εAhx-Iaa (3), Phe-(4-OMe)-His-AHNA-εAhx-Iaa-HCl (4), Boc-Phe (4-OMe)-His-AHNA-OEt (5), Boc-Phe-(4-OMe)-His-AHNA-εAhx-Iaa (6). According to the results of determination of inhibiting activity and determination of enzymatic stability it is possible to conclude that compounds 1, 2, 3 showed no inhibiting activity. We confirmed that introducing (N^Bzl) and (N^trit) substituents in histidine caused lack of activity, so it means that active renin inhibitors should contain amino acid His without any substituents. Compound 5 which contained ester bond in C-terminus was totally metabolic unstable but showed the highest activity IC₅₀ = 1.3·10⁻⁴M. The Ahx-Iaa substituent in C-terminus of compound protected C-terminus and increased the metabolic stability but reduced inhibiting activity (1).

In the search of more active and more enzymatic stable renin inhibitors we modified previous compounds introducing methyleucine instead of histidine and we incorporated AHGHA [7-(3-nitroguanidin)-3-hydroxyheptanoic acid], 8-benzyloxy-carbonylamino-3-hydroxyoctanoic acid (AAHOA) and APHPA in P₁-P₁ᵢ position. We substituted the C-terminus of three designed inhibitors with isomethylamide (Iaa) to eliminate the polarity of the mole-

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**Figure 1.** Chemical structures of 4 new renin inhibitors

Boc – Phe (4-OMe) – MeLeu – AHGHA – OEt
Compounds 1 - 4-[N-[N-[N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl]-amino]-7-[3-nitroguanidin]-3-hydroxyheptanoic acid ethyl ester

Boc – Phe (4-OMe) – MeLeu – AHGHA – εAhx – Iaa
Compounds 2 - 6-[N-[4-[N-[N-[N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl]-amino]-7-(3-nitroguanidin)-3-hydroxyheptanol-ε-aminohexanoic acid isoamylamide

Boc – Phe (4-OMe) – MeLeu – APHPA – εAhx – Iaa
Compounds 3 - 6-[N-[4-[N-[N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl]-amino]-4-amino-3-hydroxy-5-phenylpentanoil]-ε-aminohexanoic acid isoamylamide

Boc – Phe (4-OMe) – MeLeu – AAHOA – εAhx – Iaa
Compounds 4 - 6-[N-[4-[N-[N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl]-amino]-8-benzyloxy-carbonylamino-3-hydroxyoctanoic acid (AAHOA) and APHPA in P₁-P₁ᵢ position. We substituted the C-terminus of three designed inhibitors with isomethylamide (Iaa) to eliminate the polarity of the mole-

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cules and to receive a degree of hydrophobicity (6). The purpose of application of N-methyl amino acid was to obtain the inhibitors stable against chymotrypsin proteolytic activity, soluble in gastric juice as well as in lipids, and to increase inhibitory activity (5, 6, 10).

It was confirmed that methyl amino acid derivatives showed higher inhibiting activity (9, 10). Introduction of methoxy substituent in Phe in the $P_3$ position also may improve the activity (12). Compound containing MeLeu in $P_2$ position and APHPA bound to Sta showed inhibiting potency $IC_{50} = 10^{-5}$M (8). Knowing that replacement of MePhe by MeLeu improved 10 times inhibiting activity (9), we decided to design compound 3 to confirm that correlation.

The purpose of this study was to determine the inhibitory activity (in vitro) and enzymatic stability of the four new potential renin inhibitors in homogenates of body organs and body fluids (in vitro).

**EXPERIMENTAL**

**Materials and reagents**

New renin inhibitors synthesized in the Department of Drug Chemistry, Medical University of Warsaw are presented in Table 1 and Figure 1. α-Chymotrypsin from bovine pancreas was purchased from Sigma-Aldrich (USA). Angiotensinogen was purchased from Bachem (Germany) and renin from Cayman Chemical Company (USA).

**Apparatus and methods**

A Shimadzu HPLC apparatus that consisted of an LC-10AT pump and SPD-10A spectrophotometer was used with Chroma computer recorder (POL-LAB, Poland) and the Chromax 2010 software (POL-LAB, Poland).

The HPLC method for determination of compounds was used. For each of four new synthesized compounds the new HPLC method was developed. The separation was carried out in the reverse phase.
system, the flow rate was 1 mL/min. The concentration range was between 10–50 nmol/mL. The wavelength was 213 nm. The columns, mobile phases and validation parameters have been presented in Table 2.

The four HPLC methods of compounds 1–4 determination were validated. Recovery, accuracy and linearity of the analytical procedure are presented in Table 2.

Stock solutions of determined compounds were prepared by dissolving each compound in methanol. The final working concentration for the examined substances was 100 nmol/mL. The liquid-liquid extraction method was used for all biological material. Compounds were isolated with the use of diethyl ether. Compounds 3, 4 were extracted after alkalization with 0.1 mol/L NaOH (pH 10), compound 2 after alkalization with 0.1 mol/L TRIS buffer (pH 8.4) and compound 1 after acidification with 0.1 mol/L HCl (pH 3).

Determination of enzymatic stability of compounds 1–4 in vitro

The stability of all compounds in body fluids and organ homogenates was examined. The concentrations of compounds were measured at different time points during incubation in biological material. The developed HPLC method was used to determine the concentration of compounds 1–4 isolated from biological material by liquid-liquid extraction with the use of diethyl ether.

Stability determination of compounds 1–4 in liver, kidney and lung enzymes

The homogenates of body organs (40%) in 0.1 mol/L TRIS solution (pH = 8.4) were prepared. Each homogenate was spiked with each of 4 compounds and incubated at 37°C. The samples were collected at the time points 0, 30, 60, 90, 120, 150, 180 min and then isolated from homogenates with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Stability determination of compounds 1–4 in serum, gastric and intestinal juice

Each compound was dissolved in certain amount of serum or freshly prepared gastric or intestinal juice (according to USP). The solutions were incubated at 37°C and the samples were collected at the time points 0, 30, 60, 90, 120 min. The samples were isolated from body fluids with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Stability determination of compounds 1–4 in the presence of α-chymotrypsin

α-Chymotrypsin was dissolved in phosphate buffer solution pH 7.8. The solution was incubated at 37°C and the samples were collected at the time points 0, 30, 60, 90, 120 min. Then, the samples were isolated from the solution with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Determination of inhibition activity of compounds 1–4 vs. human renin

Renin inhibiting activity of the synthesized potential inhibitors was determined in vitro. The modified HPLC method of determination of

<table>
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<th>Compd. no.</th>
<th>α-Chymotrypsin</th>
<th>Serum</th>
<th>Gastric juice</th>
<th>Intestinal juice</th>
<th>Kidney homogenate</th>
<th>Lung homogenate</th>
<th>Liver homogenate</th>
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<tr>
<td>1</td>
<td>unstable</td>
<td>unstable</td>
<td>first-order reaction $t_{0.5} = 137$ min</td>
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<tr>
<td>4</td>
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<td>stable</td>
<td>stable</td>
<td>first-order reaction $t_{0.5} = 89$ min</td>
<td>stable</td>
<td>stable</td>
<td>first-order reaction $t_{0.5} = 38$ min</td>
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</table>

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Human renin IC50 (M)</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>$1.70 \times 10^{-6}$</td>
</tr>
<tr>
<td>2</td>
<td>$0.96 \times 10^{-4}$</td>
</tr>
<tr>
<td>3</td>
<td>$1.05 \times 10^{-4}$</td>
</tr>
<tr>
<td>4</td>
<td>$1.31 \times 10^{-7}$</td>
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</table>
Figure 2. Stability of compounds 1-4 in body fluids and organs (the plots of concentration vs. time)
angiotensinogen (substrate) concentration was used [column: Wild Pore C8, 15 cm×4.6 mm, mobile phase MeOH : H2O : H3PO4 (40 : 60 : 0.1, v/v/v), wavelength 213 nm] (23).

The HPLC method was modified in comparison with other method (24), used to determine the renin inhibitor activity.

The inhibition of human renin was determined after its incubation with angiotensinogen and with each of compounds 1–4. Human renin 9 mU.G. was incubated with 6 mM angiotensinogen in 30 mM citrate-phosphate buffer (pH 7.4) for 2 h at 37°C with lowering amount (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ M) of each renin inhibitor prepared in phosphoric-citric buffer pH 7.4. To stop the reaction after 2 h, the sample was immersed in boiling water for 2 min. To control the activity assay, the sample contained only human renin and angiotensinogen was prepared and the activity was measured in time 0 and after 2 h of incubation.

Figure 2. Continued
The renin inhibitory activity was designed in terms of the IC$_{50}$, which is the molar concentration of the examined inhibitor causing 50% inhibition of the control renin activity. Results are presented in Table 4.

RESULTS AND DISCUSSION

Determination of enzymatic stability of four new compounds have been performed in test in vitro. The enzymatic stability was determined in gastric and intestinal juice, serum, homogenates of liver, kidney, lungs and in the presence of α-chymotrypsin. Compound 2 and 3 were stable in all body fluids and organs. Compound 1, which contained ester bond in C-terminus was unstable, especially in liver and kidney homogenates (compound was completely decomposed after 2 h of incubation at 37°C). Decomposition in gastric juice is a first-order reaction relative to substance concentration. Introduction of ester group in P$_1$-P$_1'$ position caused that compound 4 was partly unstable. It decomposed in liver and kidney homogenates according to first-order reaction, the half-time was 38 and 89 min, respectively. Compound 4 was unstable also against α-chymotrypsin (after 2 h incubation at temp. 37°C 58% of compound decomposed). The presence of Ñ-Ahx-Iaa substituent at the C-terminus in compounds 2 and 3 made them stable in body fluids and organs.

The determination of inhibiting activity was performed by modified HPLC method. All new synthesized compounds were active at the concentration 10$^{-6}$ to 10$^{-9}$ M. It was our significant achievement that compound 3 showed the highest activity 1.05 $×$ 10$^{-9}$ M (activity of aliskiren 0.6 $×$ 10$^{-9}$ M (18)). It confirmed that proposed theoretical compound structure was proper. Compounds with C-terminus blocked with Ñ-Ahx-Iaa showed 100 times higher activity. In our previous paper (13) we observed the reverse result, so it is not possible to find the direct correlation between blocking of C-terminus and activity. In such big molecule the modification of one element may affected on physicochemical parameters and consequently on activity.

The search for other compounds with expected higher potency will be continued.

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REFERENCES


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