LOW MOLECULAR PEPTIDES AS POTENTIAL INHIBITORS OF PLASMIN

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Abstract: Ten peptides of the general formula A-Phe-Lys-X where A = H, H-D-Val, H-L-Val, H-D-Ala, H-L-Ala and X = OH, NH_2 were obtained and tested for their antiplasmin activity with the use of amidolytic test.

Keywords: plasmin inhibitor, antiamidolytic activity, low molecular peptide

Recent studies indicate that plasmin plays an important role in a variety of biological processes like wound–healing, tissue repair, cell migration (1), in pathological phenomena such as inflammation, tumor cell growth and metastasis (2, 3) in addition to its role in fibrinolysis.

ε-Aminocaproic acid (EACA) and *trans*-aminomethyl-cyclohexanecarboxylic acid (AMCHA) are clinically used as plasmin inhibitors. These compounds inhibit the fibrinolytic activity of plasmin by blocking the lysine-binding sites. Their inhibitory activity on plasmin with respect to fibrinogen, other proteins and small peptides is much weaker than towards fibrin. Because of this fact, the synthesis of active center directed low molecular inhibitors of plasmin was undertaken (4, 5). The optimal P_1-P_2 specificity for plasmin seems to be Phe-Lys (6). This cleavage sequence has been identified in many natural and synthetic substrates (6, 7). Derivatives of short peptides with C-terminal lysine carboxyl groups transformed into aldehyde (8), chloro- and fluoromethyl ketone (9, 10) or p-nitroanilide (11) are active directed inhibitors or synthetic substrates of plasmin (for example: H-Ala-Phe-Lys-X, H-D-Val-Phe-Lys-Y, where X = Cl, F, pNA; Y = Cl, pNA). The tripeptide H-D-Ala-Phe-Lys is known as a trigger in some antitumor prodrugs activable by plasmin (12-14).

It seems to be interesting to examine if short peptides with unsubstituted lysine carboxyl group can inhibit plasmin activity. Until now, only Fareed et. al. (15) reported that contraceptive peptide H-Thr-Pro-Arg-Lys-OH inhibits amidolytic activity of plasmin. Earlier we found that H-Ala-Phe-Lys-OH did not inhibit fibrinolytic and casenolytic activities of plasmin. The amidolytic activity of this compound was not examined (16).

They exist as well reports that alkyl- and benzylamides of peptides are inhibitors of plasmin (16-18). Midura-Nowaczek et al. studied H-Phe-Lys-NH-Bzl, H-Ala-Phe-Lys-NHC_7H_{15} and H-Glp-Phe-Lys-NHC_7H_{15} as potential inhibitors of plasmin. Unsubstituted amides of peptides with sequence X-Phe-Lys were not examined.

The purpose of this work was to synthesize peptides of general formula A-Phe-Lys-X, where A = H, H-D-Val, H-L-Val, H-D-Ala, H-L-Ala and X = OH, NH_2 (Table 1) and to check their influence on amidolytic activity of plasmin.

EXPERIMENTAL

Material and methods

Blocked amino acids (Z-Val-OH, Z-D-Val-OH, Z-Ala-OH, Z-D-Ala) and coupling reagent DCC (dicyclohexylcarbodiimide) were purchased

Table 1. Structure of the obtained peptides A-Phe-Lys-X.

Compound	A	X
1	H-D-Val	OH
2	H-D-Val	NH_2
3	H-L-Val	OH
4	H-L-Val	NH_2
5	H-D-Ala	OH
6	H-D-Ala	NH_2
7	H-L-Ala	OH
8	H-L-Ala	NH_2
9	H	NH_2
10	Ac	NH_2

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Table 2. Yields and physicochemical properties of tripeptides obtained.

Comp.	Yield (%)	Molecular formula	R_f: 1 2	m. p. [°C]	$[\alpha]^{20}_D$ (C=1, CH_3COOH)	^{1}H NMR (DMSO) δ ppm
1	65	$C_{20}H_{32}N_4O_4$	0.56 0.75	163	-16.67	8.86-7.64 (m, 5H, LysNH$_2$, CONH, CONH$_2$), 7.00-7.30 (m, 5H, C$_6$H$_5$), 6.48 (bs, 2H, 2 × CONH), 4.30-4.10 (m, 1H, CH$^\alpha$), 3.50-3.30 (m, 1H, CH$^\alpha$), 3.09-2.95 (m, 1H, CH$^\alpha$), 2.75-2.59 (m, 4H, LysCH$_2^\epsilon$, PheCH$_2$), 2.25-2.09 (m, 1H, ValCH) 1.80-1.10 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 0.90-0.55 (dd, J = 6.8 Hz, 6H, Val(CH$_3$)$_2$)
2	67	$C_{20}H_{33}N_5O_3$	0.77 0.87	145	-19.33	8.10-7.62 (m, 5H, LysNH$_2$, ValNH$_2$, CONH), 7.25-7.18 (m, 5H, C$_6$H$_5$), 6.95 (bs, 3H, CONH, CONH$_2$), 4.64-4.57 (m, 1H, CH$^\alpha$), 4.20-4.18 (m, 1H, CH$^\alpha$), 3.12-3.03 (m, 1H, CH$^\alpha$), 3.12-2.80 (m, 5H, LysCH$_2^\epsilon$, PheCH$_2$, ValCH), 1.83-1.18 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 0.77-0.64 (dd, J = 6.8 Hz, 6H, Val(CH$_3$)$_2$)
3	49	$C_{20}H_{32}N_4O_4$	0.56 0.75	155	-7.34	8.19-7.71 (m, 5H, LysNH$_2$ CONH, CONH$_2$), 7.02-7.32 (m, 5H, C$_6$H$_5$), 6.3 (bs, 2H, 2 × CONH), 4.35-4.24 (m, 1H, CH$^\alpha$), 3.50-3.30 (m, 1H, CH$^\alpha$), 3.09-2.95 (m, 1H, CH$^\alpha$), 2.75-2.59 (m, 4H, LysCH$_2^\epsilon$, PheCH$_2$), 1.95-1.81 (m, 1H, ValCH) 1.70-1.10 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 0.91-0.71 (dd, J = 6.8 Hz, 6H, Val(CH$_3$)$_2$)
4	45	$C_{20}H_{33}N_5O_3$	0.77 0.87	159	+22.31	8.40-7.45 (m, 4H, LysNH$_2$, 2 × CONH), 7.22-7.18 (m, 7H, NH$_2$, C$_6$H$_5$), 6.99 (bs, 2H, CONH$_2$), 4.59-4.30 (m, 1H, CH$^\alpha$), 4.22-4.16 (m, 1H, CH$^\alpha$), 3.09-2.78 (m, 5H, CH$^\alpha$, LysCH$_2^\epsilon$, PheCH$_2$), 2.56-2.50 (m, 1H, ValCH), 1.98-1.17 (m, 6H, LysCH$_2^{\beta, \gamma, \delta}$), 0.87-0.58 (dd, J = 6.8 Hz, 6H, Val(CH$_3$)$_2$)
5	65	$C_{18}H_{28}N_4O_4$	0.58 0.78	176	-6.67	8.19-7.98 (m, 3H, LysNH$_2$ CONH), 7.30-7.22 (m, 5H, C$_6$H$_5$), 6.41 (bs, 3H, CONH, CONH$_2$), 4.35-4.12 (m, 1H, CH$^\alpha$), 4.08-4.01 (m, 1H, CH$^\alpha$), 3.98-3.86 (m, 1H, CH$^\alpha$), 3.02-2.73 (m, 4H, LysCH$_2^\epsilon$, PheCH$_2$), 1.52-1.12 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 1.19-1.15 (d, J = 6.8 Hz, 3H, AlaCH$_3$)
6	74	$C_{18}H_{29}N_5O_3$	0.56 0.67	133	+3.33	8.13-7.74 (m, 4H, LysNH$_2$, 2 × CONH), 7.21-7.15 (m, 5H, C$_6$H$_5$), 6.99 (bs, 2H, CONH$_2$), 4.59-4.55 (m, 1H, CH$^\alpha$), 4.18-4.16 (m, 1H, CH$^\alpha$), 3.24-3.17 (m, 1H, CH$^\alpha$), 3.09-2.69 (m, 4H, LysCH$_2^\epsilon$, PheCH$_2$), 1.62-1.29 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 1.03-0.99 (d, J = 6.8 Hz, 3H, AlaCH$_3$)
7	71	$C_{18}H_{28}N_4O_4$	0.58 0.78	174 160-163 lit. [16]	-2.66 +4.4[a] +4.1 lit. (16)	8.21-7.92 (m, 3H, LysNH$_2$ CONH), 7.22-7.04 (m, 5H, C$_6$H$_5$), 6.79-6.60 (m, 1H, CONH), 6.53-6.27 (m, 2H, CONH$_2$), 4.33-4.07 (m, 1H, CH$^\alpha$), 4.09-3.92 (m, 2H, 2xCH$^\alpha$), 3.17-2.72 (m, 4H, LysCH$_2^\epsilon$, PheCH$_2$), 1.90-1.24 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 1.18-1.09 (d, J = 6.8 Hz, 3H, AlaCH$_3$)
8	75	$C_{18}H_{29}N_5O_3$	0.56 0.67	183	-8.67	8.76 (bs, 2H, LysNH$_2$ AlaNH$_2$), 8.36-8.32 (m, 1H, CONH), 7.40 (bs, 2H, CONH$_2$), 7.26-7.14 (m, 5H, C$_6$H$_5$), 7.02 (bs, 1H, CONH), 4.59-4.55 (m, 1H, CH$^\alpha$), 4.20-4.10 (m, 1H, CH$^\alpha$), 3.69-3.63 (m, 1H, CH$^\alpha$), 3.19-2.69 (m, 5H, LysCH$_2^\epsilon$, PheCH$_2$), 1.68-1.29 (m, 6H, Lys CH$_2^{\beta, \gamma, \delta}$), 1.03-0.99 (dd, J = 6.8 Hz, 3H, AlaCH$_3$)

Table 2. Cont.

| 9 | 78 | $C_{15}H_{24}N_4O_2 \times$ 2 CH$_3$COOH | 0.63 0.79 | 171 | -12.1 | 8.00 (d, J = 7.9Hz, 1H, CONH), 7.27-7.24 (m, 5H, C$_6$H$_5$), 6.12-5.70 (m, 8H, Lys NH$_3^+$, PheNH$_3^+$, CONH$_2$), 4.50-4.42 (m, 1H, CH$^\alpha$), 4.16-4.04 (m, 1H, CH$^\alpha$), 3.03-2.98 (m, 2H, LysCH$_2^\epsilon$), 2.80-2.70 (m, 2H, PheCH$_2$), 1.82 (s, 3H, CH$_3$COO$^-$), 1.73-1.29 (m, 6H, LysCH$_2$ $^{\beta, \gamma, \delta}$) |
| 10 | 77 | $C_{17}H_{27}N_4O_3 \times$ CH$_3$COOH | 0.79 0.84 | 201 | -2.67 | 8.17-7.79 (m, 9H, CONH, Lys NH$_3^+$, PheNH$_3^+$, CONH$_2$), 7.27-7.25 (m, 5H, C$_6$H$_5$), 4.50-4.42 (m, 1H, CH$^\alpha$), 4.16-4.04 (m, 1H, CH$^\alpha$), 3.03-2.97 (m, 2H, LysCH$_2^\epsilon$), 2.79-2.69 (m, 2H, PheCH$_2$), 1.86 (s, 3H, CH$_3$COO$^-$), 1.76 (s, 3H, Ac CH$_3$), 1.67-1.30 (m, 6H, LysCH$_2$ $^{\beta, \gamma, \delta}$) |

[a] C =1, MeOH

Table 3. Inhibition of amidolytic activity of plasmin.

No.	Compound	IC$_{50}$ (M) S-2251
1	H-D-Val-Phe-Lys-OH	> 0.02
2	H-D-Val-Phe-Lys-NH$_2$	0.012
3	H-L-Val-Phe-Lys-OH	0.01
4	H-L-Val-Phe-Lys-NH$_2$	0.001
5	H-D-Ala-Phe-Lys-OH	0.014
6	H-D-Ala-Phe-Lys-NH$_2$	n.i.
7	H-L-Ala-Phe-Lys-OH	0.006
8	H-L-Ala-Phe-Lys-NH$_2$	> 0.02
9	H-Phe-Lys-NH$_2$	0.001
10	Ac-Phe-Lys-NH$_2$	0.01

n.i. = no inhibition was observed at maximum concentration (0.02M)

from Merck. HOBt (1-hydroxybenzotriazole), H-Lys(Z)-OMe × HCl (Z = benzyloxycarbonyl) and Boc-Phe-ONp (Boc = t-butoxycarbonyl, ONp = 4-nitrophenyl ester) were purchased from Fluka. Plasmin, S-2251 (H-D-Val-Leu-Lys-pNA) (Chromogenix), and bovine fibrinogen (Lubelska Wytwórnia Szczepionek, Lublin, Poland), were used in enzymatic investigations.

Organic solutions were dried over anhydrous MgSO$_4$. Reactions were monitored and homogeneity of products was examined using the silica gel plates (Kiesegel 60 F$_{254}$, Merck) using following systems: 1: benzene/methanol/acetic acid (12:5:1, v/v/v), 2: ethanol/water/25% ammonia solution (18:0.5:0.5, v/v/v). Spots were visualized at the beginning with iodine and after removing off the iodine with ninhydrin. The melting points were determined on Böetius block and are uncorrected. The specific optical rotations were measured with a polarimeter (Optical Activity LTD AA-10R). ^{1}H-NMR spectra were recorded with 200 MHz Bruker AC 200F spectrometer. Elemental analyses were performed on a Perkin-Elmer analyzer and results were within ± 0.4% of theoretical values.

Synthesis

Classical coupling techniques were used to prepare all peptides. The synthesis of peptides was carried out using DCC with addition of HOBt. In the first step HCl·H-Lys(Z)-OMe was coupled with Boc-Phe-ONp. After the Boc removal under acidic conditions, the dipeptide HCl·H-Phe-Lys(Z)-OMe was coupled with the third amino acid (Z-D-Val-OH, Z-L-Val-OH, Z-D-Ala-OH, Z-L-Ala-OH). Methyl ester moiety of tripeptides was removed by alkaline hydrolysis in methanol. Amides of peptides were prepared from the peptide methyl esters using methanol saturated with NH$_3$. Benzyloxycarbonyl group was removed by catalytic hydrogenation with the use of Pd/C. Methanol with small addition of water (tripeptides) or acetic acid (dipeptides) was used as a solvent t-Butoxycarbonyl group was removed with the use of methanol saturated with NH$_3$. Final and intermediate compounds were purified by crystallization from methanol with water and washed with dry diethyl ether. H-L-Ala-Phe-Lys-OH was described in the literature (16) , but there were given only melting point and specific rotation. The results of synthesis are given in Table 2.

Enzymatic investigations

The determination of amidolytic activity was performed with use of synthetic substrate S-2251 as described previously (18). Detailed description of the method is given below: 0.2 cm^3 of the examined preparation (0.15 M NaCl as control), tris buffer – 0.5 cm^3 (pH = 7.4) and 0.1 cm^3 of plasmin (0.4 units/cm^3) were added. The mixture was incubated at 37°C for 3 min then synthetic substrate S-2251 solution (0.2 cm^3, 3 mM/dm^3) in the same buffer

was added. After 20 min incubation, the reaction was stopped by addition of 0.1 cm^3 of 50% acetic acid and the absorbance of released p-nitroaniline was measured at 405 nm. Every value represents the average of triplicate determination. IC$_{50}$ value was considered as the concentration of inhibitor which decreased the absorbance at 405 nm by 50% compared with the absorbance measured under the same conditions without inhibitor. The results are given in Table 3.

RESULTS AND DISCUSSION

According to the results obtained, the examined peptides inhibit amidolytic activity of plasmin. The absence of inhibitory activity of **6** (H-D-Ala-Phe-Lys-NH$_2$) is only one exception. L-amino acid in P$_3$ position of tripeptides and their amides results in more effective inhibition than D-enantiomer. This result agrees with our earlier research on tripeptide methyl ketones (19). According to the literature, tripeptide synthetic substrates with D-configuration in P$_3$ have better affinity to plasmin (7). However, among the best irreversible inhibitors of plasmin with chloromethyl structure, there are compounds with D- (H-D-Val-Phe-Lys-CH$_2$Cl) (11) and L-configuration (H-Ala-Phe-Lys-CH$_2$Cl) in P$_3$ (10). The problem of the preferable P$_3$ configuration of reversible inhibitors of plasmin with tripeptide structure needs further investigations.

One of the best inhibitors of plasmin is dipeptide **9** (H-Phe-Lys-NH$_2$). Acetylation of free amine group of Phe in P$_2$ (compound **10**) reduces significantly inhibitory activity. It seems that basic importance for inhibition of plasmin activity has the dipeptide fragment Phe-Lys, and position P$_3$ is of smaller significance.

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