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Short peptides containing L-lysine and ε-aminocaproic acid as potential plasmin inhibitors

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Eight short peptides containing L-lysine and ε-aminocaproic acid were obtained and their effect on the amidolytic activities of plasmin, thrombin and trypsin was examined. Tripeptide amide Boc-EACA-L-Lys-EACA-NH_2 was the most effective and specific plasmin inhibitor.

1. Introduction

Plasmin, a key enzyme for fibrynolysis, plays an important role in various biological processes, i.e. wound healing, tissue repair and cell migration (Hervio et al. 2000). It is also important in such pathological phenomena as inflammation, tumour cell growth and metastasis (Wanaka et al. 1996).

ε-Aminocaproic acid (EACA) and trans-aminomethylcyclo–hexanecarboxylic acid (AMCHA) - lysine analogs with antifibrinolytic activity are used clinically as plasmin inhibitors.

The aim of this research has been to obtain an active-centre directed inhibitor of plasmin which influences not only fibrinolysis but also amidolysis and proteolysis. The inhibitor which controls such plasmin activity would be very useful in determining the physiological and pathological function of this enzyme, and in treating plasmin-associated disorders. Plasmin has P1 preference for lysine (Backes et al. 2000). The derivatives of this amino acid have been widely examined as potential synthetic substrates and inhibitors of the enzyme. The plasmin inhibitors: ε-aminocaproyl-L-lysine (Fuji et al. 1972) and the substituted anilide of 5-aminopentanoil-L-lysine ($NH_2(CH_2)_4$-CO-Lys-NH-C_6H_4-CO-C_6H_5), determined as OS-175 (Okamoto et al. 1987), belong to this group of compounds.

Table 1: Structure of peptides obtained

	A_1-L-Lys(X)-Y		
Compd.	A_1	X	Y
1	Boc-EACA	Z	EACA-NH_2
2	Boc-EACA	H	EACA-NH_2
3	H-EACA	H	EACA-NH_2
4	Boc-EACA	Z	EACA-OCH_3
5	Boc-EACA	H	EACA-OCH_3
6	H-EACA	H	EACA-OCH_3
7	Boc	Z	EACA-NH_2
8	Boc	H	EACA-NH_2

During our earlier investigations on the active-centre directed plasmin inhibitors, a series of lysine amides (Midura-Nowaczek et al. 2008) and dipeptide derivatives with C-terminal lysine cyclohexyl, benzyl and hexyl amides were examined. Compounds with cadaverine residue connected with the lysine were also tested (Midura-Nowaczek et al. 2003, 2006). The obtained results show that some of these compounds inhibited the amidolytic and fibrinolytic activity of plasmin.

Table 2: Inhibition of enzyme amidolytic activity

Compound	Plasmin (S-2251)	Thrombin (S-2238)	Trypsin (Bzl-Arg-pNA·HCl)
	IC_{50} (mM)		
Boc-EACA-Lys(Z)-EACA-NH_2	20 ± 1.4	n.i.	>20
Boc-EACA-Lys-EACA-NH_2	0.02 ± 0.0014	n.i.	>20
H-EACA-Lys-EACA-NH_2	9.0 ± 0.63	7.5 ± 0.53	>20
Boc-EACA-Lys(Z)-EACA-OMe	>20	n.i.	n.i.
Boc-EACA-Lys-EACA-OMe	0.6 ± 0.042	n.i.	>20
H-EACA-Lys-EACA-OMe	0.8 ± 0.056	12.0 ± 0.84	11 ± 0.77
Boc-Lys(Z)-EACA-NH_2	8.0 ± 0.56	n.i.	n.i.
Boc-Lys-EACA-NH_2	n.i.	n.i.	n.i.

n.i. = no inhibition was observed in maximum concentration (0.02 M)
Boc = *t*-butoxycarbonyl
Z = benzyloxycarbonyl

Table 3: Analytical data of obtained compounds

No	Compound	Yield (%)	R_f: 1 2	m.p. [°C]	$[\alpha]_D^{20}$ (C = 1, MeOH)	^{1}H NMR (DMSO)
1	Boc-EACA-(Z)-Lys-EACA-NH_2	78.8	0.61 0.87	109	−17.2	7.92-7.78 (m, 2H, NH), 7.42-7.3 (m, 5H, C_6H_5Z), 7.3-7.15 (m, 2H, NH), 6.8-6.65 (m, 2H, NH), 4.99 (s, 2H, CH_2Z), 4.23-4.09 (m, 1H, CH^{α}Lys), 3.08-2.17 (m, 6H, $CH_2{}^{\varepsilon}$Lys, 2x$CH_2{}^{\varepsilon}$EACA), 2.12-1.97 (m, 4H, 2x$CH_2{}^{\alpha}$EACA), 1.58-1.12 (m, 27H, Boc, 6xCH_2EACA, 3xCH_2Lys)
2	Boc-EACA-Lys-EACA-NH_2	46.4	0.77 0.84	120	−26.6	7.89-7.77 (m, 2H, NH), 7.28-7.13 (m, 2H, NH), 6.8-6.65 (m, 2H, NH), 4.23-4.09 (m, 1H, CH^{α}Lys), 3.11-2.16 (m, 6H, $CH_2{}^{\varepsilon}$Lys, 2x$CH_2{}^{\varepsilon}$EACA), 2.14-1.99 (m, 4H, 2x$CH_2{}^{\alpha}$EACA), 1.58-1.11 (m, 27H, Boc, 6xCH_2EACA, 3xCH_2Lys)
3	H-EACA-Lys-EACA-NH_2	61.5	0.67 0.71	86.5	+6.2	7.90-7.76 (m, 2H, NH), 7.28-7.13 (m, 2H, NH), 6.82-6.65 (m, 2H, NH), 4.21-4.11 (m, 1H, CH^{α}Lys), 3.06-2.15 (m, 6H, $CH_2{}^{\varepsilon}$Lys, 2x$CH_2{}^{\varepsilon}$EACA), 2.12-1.97 (m, 4H, 2x$CH_2{}^{\alpha}$EACA) 1.59-1.08 (m, 18H, 6xCH_2EACA, 3xCH_2Lys)
4	Boc-EACA-(Z)-Lys-EACA-OMe	82.7	0.68 0.82	113	−12.3	7.94-7.7 (m, 2H, NH), 7.4-7.28 (m, 5H, C_6H_5Z), 6.74 (t, 1H, NH), 4.99 (s, 2H, CH_2Z), 4.22-4.08 (m, 1H, CH^{α}Lys), 3.56 (s, 3H, OMe), 3.07-2.79 (m, 6H, 2x$CH_2{}^{\varepsilon}$EACA, $CH_2{}^{\varepsilon}$Lys,), 2.26 (t, 2H, $CH_2{}^{\alpha}$EACA), 2.08 (t, 2H, $CH_2{}^{\alpha}$EACA), 1.58-1.12 (m, 27H, Boc, 6xCH_2EACA, 3xCH_2Lys)
5	Boc-EACA-Lys-EACA-OMe	50.7	0.71 0.79	123	−18.7	7.94-7.7 (m, 2H, NH), 6.74 (t, 1H, NH), 4.22-4.08 (m, 1H, CH^{α}Lys), 3.58 (s, 3H, OMe) 3.11-2.77 (m, 6H, 2x$CH_2{}^{\varepsilon}$EACA, $CH_2{}^{\varepsilon}$Lys,), 2.26 (t, 2H, $CH_2{}^{\alpha}$EACA), 2.11 (t, 2H, $CH_2{}^{\alpha}$EACA), 1.59-1.08 (m, 27H, Boc, 6xCH_2EACA, 3xCH_2Lys)
6	H-EACA-Lys-EACA-OMe	72.3	0.57 0.48	oil	+3.2	7.92-7.74 (m, 2H, NH), 6.72 (t, 1H, NH), 4.19-4.07 (m, 1H, CH^{α}Lys), 3.56 (s, 3H, OMe) 3.07-2.79 (m, 6H, 2x$CH_2{}^{\varepsilon}$EACA, $CH_2{}^{\varepsilon}$Lys,), 2.26 (t, 2H, $CH_2{}^{\alpha}$EACA), 2.08 (t, 2H, $CH_2{}^{\alpha}$EACA), 1.59-1.12 (m, 18H, 6xCH_2EACA, 3xCH_2Lys)
7	Boc-(Z)-Lys-EACA-NH_2	42.9	0.60 0.72	119	−15.1	7.72 (t, 1H, NH), 7.42-7.28 (m, 5H, C_6H_5Z), 7.28-7.15 (m, 2H, NH), 6.75-6.6 (m, 2H, NH), 4.99 (s, 1H, CH_2Z), 3.85-3.72 (m, 1H, CH^{α}Lys), 3.05-2.9 (m, 4H, $CH_2{}^{\varepsilon}$Lys, $CH_2{}^{\varepsilon}$EACA), 2.02 (t, 2H, $CH_2{}^{\alpha}$EACA) 1.55-1.15 (m, 21H, Boc, 3xCH_2EACA, 3xCH_2Lys)
8	Boc-Lys-EACA-NH_2	37.4	0.33 0.64	oil	−5.2	7,72 (t, 1H, NH), 7,28-7,15 (m, 2H, NH), 6,75-6,6 (m, 2H, NH), 3,85-3,72 (m, 1H, CH^{α}Lys), 3,05-2,9 (m, 4H, $CH_2{}^{\varepsilon}$Lys, $CH_2{}^{\varepsilon}$EACA), 2,02 (t, 2H, $CH_2{}^{\alpha}$EACA), 1,57-1,15 (m, 21H, Boc, 3xCH_2EACA, 3xCH_2Lys)

2. Investigations and results

In the search for new low molecular plasmin inhibitors with a simple and easy to synthesize structure, we obtained eight short peptides containing L-lysine and ε-aminocaproic acid. Every synthesized dipeptide or tripeptide was transformed into methyl ester or unsubstituted amide. Some of the compounds also have protected amino groups (Table 1). The effect of the obtained short peptides on the amidolytic activities of plasmin, thrombin and trypsin was

determined as the IC_{50} values. The results are presented in Table 2.

3. Discussion

According to the results obtained, two ε-aminocaproic acid residues and one residue of lysine are necessary in the plasmin inhibitor structure. The dipeptides are practically inactive. Only compound **7** was a weak inhibitor of plasmin. The amid of tripeptide with Boc-substituted N-terminal amino group of EACA (**2**) was the most selective inhibitor of the amidolytic activity of plasmin (Table 2). Its inhibitory activity was similar to the value obtained for OS-175 (IC_{50} = 16 μM in the amidolytic test; Okamoto et al. 1987). The derivative with C-terminal amide residue and the unsubstituted N-terminal amino group of EACA (**3**) was a weak inhibitor of plasmin and thrombin. Practically no difference in the plasmin activity inhibition was observed in the methyl esters of tripeptides with Boc substituted (**5**) and unsubstituted (**6**) N-terminal amino groups of EACA (Table 2). However, the compound with Boc group (**5**) seems to be a more selective inhibitor. The substitution of N^{ε}-amino group of lysine in tripeptides results in the disappearance of the inhibitory activity or its drastic decrease. Our results suggest that the simple derivatives of lysine containing EACA may be efficient and selective active-centre directed inhibitors of plasmin.

4. Experimental

4.1. Synthesis of the compounds

Classical coupling techniques were used to prepare all peptides. The *t*-butoxycarbonyl group was removed with the use of HCl saturated solvents. The benzyloxycarbonyl group was removed by catalytic hydrogenation. Organic solutions were dried over anh. $MgSO_4$. The homogeneity of the products was examined on silica gel plates (Kiesegel 60 F_{254}, Merck) using the following solvent systems: 1: benzene/methanol/acetic acid (12:5:1), 2: ethanol/water/25% ammonia solution (18:0.5:0.5), 3: butanol/acetic acid/water (4:2:5), 4: chloroform/acetone (7:1).
The spots were visualized with iodine and ninhydrin. The melting points were determined on a Boetius block and are uncorrected. The specific optical rotation was 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 Perkin-Elmer analyser and results were within ±0.4% of theoretical values. The analytical data are given in Table 3.

4.2. Enzymatic investigations

Plasmin, S-2251 (H-D-Val-L-Leu-L-Lys-pNA·2HCl) and S-2238 (H-D-Phe-Pip-Arg-pNA·2HCl) (Chromogenix); trypsin and Bzl-L-Arg-pNA·HCl (Sigma); thrombin (Lubelska Wytwórnia Szczepionek, Lublin, Poland).

The determination of amidolytic activity was performed with standard methods. The detailed description of the method used in the test is given below: to 0.2 ml of examined preparation (as control 0.15 M NaCl), buffer and 0.1 ml of enzyme solution was added. The mixture was incubated at 37 °C for 3 min then synthetic substrate solution in the same buffer was added. After incubating for 20 min, 0.1 ml of 50% acetic acid was added to stop the reaction and the absorbance of the released p-nitroaniline was measured at 405 nm. Every value represents the average of triplicate determination ±SD. IC_{50} value was considered to be the concentration of the inhibitor, which decreased the absorbance at 405 nm by 50%, compared with the absorbance measured under the same conditions without the inhibitor.

a. tris buffer – 0.5 ml (pH = 7.4);
enzyme: plasmin (0.4 units/ml)
synthetic substrate: S-2251 (0.2 ml, 3 mM/l)
b. tris buffer – 0.5 ml (pH = 8.4)
enzyme: thrombin (1 units/ml)
synthetic substrate: S-2238 (0.2 ml, 0.75 mM/l)
c. borane buffer – 0.5 ml (pH = 7.5)
enzyme: trypsin (0.4 units/ml)
synthetic substrate: Bzl-L-Arg-pNA·HCl (0.2 ml, 8 mM/l)

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