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EFFECT OF SHORT PEPTIDES CONTAINING LYSINE AND ε-AMINOCAPROIC ACID ON FIBRINOLYTIC ACTIVITY OF PLASMIN AND TOPOISOMERASE II ACTION ON SUPERCOILED DNA

KRYSTYNA MIDURA-NOWACZEK*, MACIEJ PURWIN, AGNIESZKA MARKOWSKA, DANUTA DROZDOWSKA and MAGDALENA BRUZGO

Department of Organic Chemistry, Medical University of Białystok, Kiliński St. 1, 15-089 Białystok, Poland

Abstract: Effects of eight short peptides containing lysine and ε-aminocaproic acid (EACA) on prolongation of the clot lysis time, as well as hemolytic and antibacterial activities were investigated. Interaction with plasmids pBR322 and pUC19 with the use of ethidium bromide assay and determination of influence on the activity of topoisomerase I and II were also tested. Examined compounds inhibited fibrinolytic activity of plasmin and five of them were more active than EACA. Amides of dipeptides were most active antifibrinolytics (IC_{50} < 0.2 mM). According to the obtained data, the significant inhibition of fibrinolytic activity of plasmin was not associated with hemolytic effects. Examined compounds did not show antibacterial activity (MIC > 512 mg/L). DNA binding effects determined with the use of ethidium bromide were weak for all peptides and similar to those observed with EACA. Six compounds inhibited topoisomerase II action on supercoiled DNA.

Keywords: peptides, ε-aminocaproic acid, antifibrinolytic, topoisomerase, DNA binding

Plasmin, a key enzyme for fibrinolysis, plays an important role in various biological processes, i.e., wound healing, tissue repair and cell migration. It is also important in such pathological phenomena as inflammation, tumor, cell growth and metastasis. ε-Aminocaproic acid (EACA) and trans-aminomethylcyclohexanecarboxylic acid (AMCHA) – lysine analogs with antifibrinolytic activity were used clinically as plasmin inhibitors. Those ω-amino acids exhibited an inhibitory effect by blocking the lysine binding sites (LBS) of the enzyme. Their inhibitory activity on plasmin with respect to fibrinogen and other proteins was much weaker than towards fibrin. Plasmin has P1 preference for lysine (1). The derivatives of those amino acids had widely been examined as potential synthetic substrates and active center directed inhibitors of the enzyme, which influenced not only fibrinolysis but also amidolysis and proteolysis. The inhibitor that controls such plasmin activity would be very useful in determining the physiological and pathological functions of this enzyme, and in treating plasmin-associated disorders.

Earlier, we obtained eight short peptides containing lysine and ε-aminocaproic acid with general formula A-Lys(X)-B, where A = H, Boc, H-EACA, Boc-EACA; B = NH_2, EACA-NH_2, EACA-OMe; X = H, Z; and examined their effect on amidolytic activity of plasmin, thrombin and trypsin (2). The aim of this research was to obtain an active center directed inhibitor of plasmin with simple structure which can be easy to synthesize. According to the results obtained, two ε-aminocaproic acid residues and one residue of lysine were necessary in the plasmin inhibitor structure. The amide of tripeptide with Boc-EACA-Lys-EACA-NH_2 was the most active and selective inhibitor of the amidolytic activity of plasmin. Its inhibitory activity (IC_{50} = 20 μM) was similar to the value obtained for the substituted anilide of 5-aminopentanoyl-L-lysine [$NH_2(CH_2)_4$-CO-Lys-NH-C_6H_4-CO-C_6H_5], named as OS-175 (IC_{50} = 16 μM, in the amidolytic test) (3).

Certain low molecular plasmin inhibitors possessed pro-apoptotic action (4, 5) or showed antitumor activity (6-8). Antibacterial activity of some ω-aminoacyl derivatives of amino acids is well known

* Corresponding author: e-mail: krystyna.midura-nowaczek@umb.edu.pl; phone +48 85 748 5687

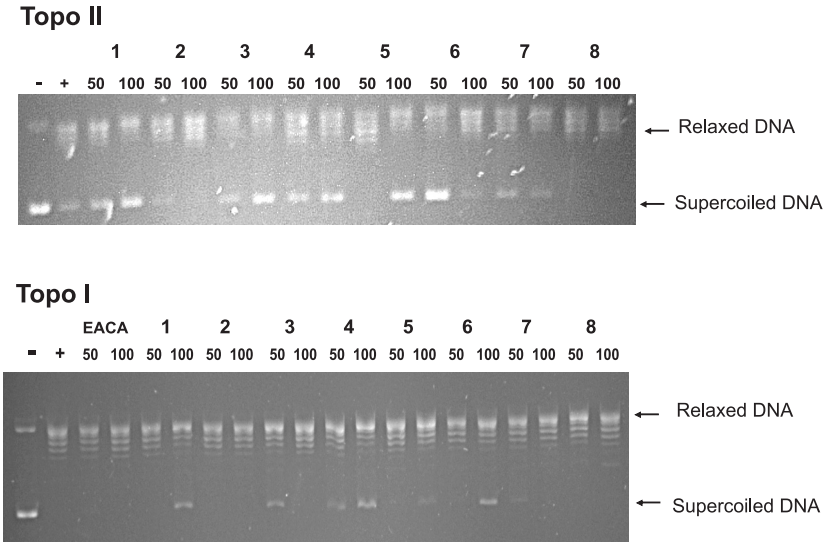

Figure 1. Effect of topoisomerase I and II on relaxation of supercoiled DNA

(9, 10). During our earlier examination of synthetic minor groove binders with oligopeptide structure (lexitropsins), we observed that these compounds could inhibit the activity of topoisomerases I and II and showed DNA binding activity. Some of them were inhibitors of amidolytic activity of trypsin-like serine proteases: plasmin and urokinase (11).

It seemed interesting to examine the activity of the inhibitors of amidolytic activity of plasmin (2) on fibrinolytic action of the enzyme and check, if they could show antitumor or antibacterial activity. In the present paper, we checked the possibility to prolong the clot lysis time, hemolytic and antibacterial effect of the tested compounds and their influence on the activity of topoisomerase I and II, as well as interaction with plasmids pBR322 and pUC19 as determined with the use of ethidium bromide (EtBr) assay.

EXPERIMENTAL

Antifibrinolytic activity

The mixture of 0.2 mL of plasmin (0.2 u/mL) and 0.1 mL of thrombin (0.2 u/mL) was added to 0.1 mL of 0.5% fibrinogen solution. Then, 0.1 mL of the examined compound in concentration of 1-100 mM* (in control 0.15 M NaCl) was added and the clot lysis time was measured. All the tests were performed three times and the mean values of measurements were used for determination of IC_{50}.

The IC_{50} value was taken as the concentration of inhibitor that prolonged the time of fibrinolysis twofold in comparison with that without inhibitor.

Antibacterial activity

The antibacterial activity of examined compounds was determined by establishing their minimal inhibitory concentration (MIC) against strains of microorganisms: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* as described by National Committe for Clinical Laboratory (12).

Hemolytic activity

Pig's fresh red blood cells (p-RBC) were washed three times with PBS (35 M phosphate buffer/0.15 mM NaCl, pH 7.4) and were centrifuged at 1000 × g for 10 min to remove plasma and the buffy coat. The peptides in concentrations up to 1000 μg/mL were incubated with the erythrocyte suspension for 1 h at 37°C (the final erythrocyte concentration was 5% v/v). After the centrifugation, 100 μL of the supernatant was transferred into sterilized 96-well plates, where hemoglobin release was monitored with the use of the Infinite M200 plate reader by measuring the absorbance at 414 nm. Zero hemolysis (blank), hemolysis with amyloride as ref-

* the examined compounds were dissolved in 0.15 M NaCl and pH of solution was adjusted to 7.4

erence compound for synthesized peptides and 100% hemolysis which consisted of p-RBC suspended in PBS and 0.1% Triton-X-100 were determined, respectively. The percentage of hemolysis was calculated with the following formula:

%hemolysis = (Abs_{414} of the peptide solution in PBS/Abs_{414} in 0.1% Triton-X-100 in PBS) × 100.

Ethidium bromide assay

Each well of 96-well plate was loaded with Tris buffer containing EtBr (0.1 M Tris, 1 M NaCl, pH 8, 0.5 mM EtBr final concentration, 100 µL). The water solution (0.05 µg/µL) of 15 µg of plasmid pBR322 (or pUC19, respectively) was added to each well. Then, pentamidine, netropsin or compound **1-8** (1 µL of a 1 mM solution in water, 10 µM final concentrations) was added to each well. After incubation at 25°C for 30 min, fluorescence of each well was read on fluorescence spectrophotometer Infinite M200 TECAN (ex. 546 nm, em. 595 nm) in duplicate experiments with two control wells (no drug = 100% fluorescence, no DNA = 0% fluorescence). Fluorescence readings are reported as % fluorescence relative to the controls.

Relaxation assay of topoisomerase I and II

Native pBR322 plasmid DNA (0.20 µg) was incubated with 4 units of human topoisomerase I (reaction buffer: 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5 M NaCl, 1 mM dithiothreitol) or topoisomerase II (reaction buffer: 10 mM Tris-HCl (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA and 15 µg/mL bovine serum albumin) in the absence or presence of varying concentrations of the test compounds (50 and 100 µM) in a final volume of 10 µL. The mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 2 µL of 10% SDS. The reaction mixture was subjected to electrophoresis (3 h, 90 V) through a 1.0% agarose gel in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained for 30 min with EtBr solution (0.5 µg/mL).

Table 1. Structures of examined peptides.

A_1-L-Lys(X)-Y			
Compound	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

Boc = *tert*-butoxycarbonyl; Z = benzyloxycarbonyl;
EACA = ε-aminocaproic acid (6-aminohexanoic acid).

Table 2. Antifibrinolytic activity of EACA and compounds **1-8**.

Compound	**EACA**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
IC_{50} (mM)	2	20	19	8.1	1.2	1.4	18	< 0.2	< 0.2

Table 3. DNA binding effect of EACA and compounds **1-8** (as % of fluorescence).

Compound	**EACA**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
pBR322	90.13	86.31	83.40	91.29	85.06	84.65	75.93	81.33	88.80
pUC19	82.70	96.20	86.50	76.80	79.75	80.17	76.80	84.81	81.01

The DNA was visualized using 312 nm wavelength transilluminator and photographed under UV light. For the quantitative determination of topoisomerase activity, area representing supercoiled DNA, migrating as a single band at the bottom of gel was measured using InGenius gel documentation and analysis system (TK Biotech). The concentrations of the compounds that converted 50% of the supercoiled DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

RESULTS AND DISCUSSION

The examined peptides showed antifibrinolytic activity and five of them (**3-5, 7, 8**) were more active than EACA (Tab. 2) but there was poor dependence between this effect and observed earlier inhibition of plasmin amidolytic activity. Compound **2**, the best inhibitor of amidolytic activity of plasmin (IC_{50} = 20 μM) (2), proved to be very weak antifibrinolytic (IC_{50} = 19 mM, Tab. 2) but compound **8** with significant possibility to prolongation of the clot lysis time (IC_{50} < 0.2 mM, Tab. 2) did not influence amidolytic activity of plasmin, thrombin or trypsin (2). High antifibrinolytic activities of dipeptides **7** and **8** were unexpected. Compound **8** was not active center directed inhibitor of plasmin and inhibitory effect of **7** on amidolytic activity of the enzyme was low (2). They couldn't block LBS of plasmin, because they did not have unsubstituted amino and carboxyl group in distance close to 7Å in their structures, as it is in the case of EACA and AMCHA. The prolongation of the clot lysis time was probably the result of interaction of examined compounds with fibrin clot.

In the examination of hemolytic effect, the results showed that the concentration up to 1000 μg/mL of the peptides **2, 3** and **5-8** did not lyse erythrocytes. In the case of the fully protected tripeptides **1** and **4** slight hemolytic activity (17.1 and 13.8%, respectively) was observed. According to the obtained data, the significant inhibition of amidolytic (**2**) or fibrinolytic (**7**, **8**) activity of plasmin was not associated with hemolytic effect. The examined compounds did not show antibacterial activity (MIC > 512 mg/L).

DNA binding effects determined with the use of EtBr were weak for all peptide products and similar to those observed with EACA. Only in the case of tripeptides derivatives with unsubstituted amino groups (compounds **3** and **6**) certain effect was observed (Tab. 3). The influence of tested substances on topoisomerase I activity was practically not observed, but compounds **1** and **3**-**7** inhibited topoisomerase II action on supercoiled DNA (Fig. 1). Amide tripeptide **2** and dipeptide **8** with unsubstituted lysine Í-amino group and protected another amino group did not blocked the topoisomerase activity. The mechanism of action of tested peptides needs further studies.

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