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Liporetro-D-peptides - A novel class of highly selective thrombin inhibitors

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ABSTRACT

Introduction: Plasma serine protease thrombin plays a key role in coagulation, haemostasis and thromboembolic diseases. Direct thrombin inhibitors could be beneficial for future anticoagulant therapy. We have synthesized and studied liporetro-D-peptides - efficient thrombin inhibitors resistant to enzymatic degradation.
Materials and Methods: Compounds X-D-Arg-D-Phe-OMe, where X = residue of lauric or myristic acid or 9-fluorenylmethoxycarbonyl, have been synthesized by conventional peptide synthesis in solution and their comparative inhibitory analysis in relation to thrombin, factor X, plasmin and trypsin has been conducted.
Results: Modification of the synthetic liporetro-D-peptides with the myristic acid residue was the most successful one. This modification has dramatically increased the inhibition efficacy ($Ki = 0{,}17\ \mu M$) and selectivity toward the chosen target enzyme, thrombin, in comparison to factor X, plasmin and trypsin (more than 600, 900, and 5000-fold, respectively).
Conclusions: Our findings establish an important role of the fatty moiety in the structure of peptide inhibitors with regards to their potency and selectivity toward thrombin.

Introduction

Thromboembolic diseases such as deep vein thrombosis, myocardial infarction, unstable angina, pulmonary embolism, and ischaemic stroke are a major cause of morbidity and mortality in the industrialized world. From a survey of the recent literature, it is clear that the discovery of a highly selective, potent, and orally bioavailable inhibitors of thrombin, a serine protease which is the main target for antithrombotic drug development, is a top priority for many research laboratories throughout the world. However, there are many limitations of approved anticoagulants. Development of conceptually new inhibitors is essential, as current drug candidates have failed due to poor selectivity, inherent toxicity, poor metabolic stability, rapid elimination from the blood, low anticoagulant activity, poor oral bioavailability and a narrow therapeutic index with the risk of bleeding complications [1,2].

Thrombin (EC 3.4.21.5), is a multifunctional serine protease that plays a central role in haemostasis [3]. Generated in response to vascular injury, thrombin converts soluble fibrinogen into insoluble fibrin. Thrombin also serves as a potent platelet agonist and amplifies its own generation by feedback activation of several steps in the coagulation cascade. Once bound to thrombomodulin, its endothelial cell receptor, thrombin assumes anticoagulant and antifibrinolytic properties. The anticoagulant properties reflect the capacity of the thrombin/thrombomodulin complex to activate protein C, which serves as an anticoagulant by downregulating thrombin generation. Activation of the thrombin activatable fibrinolysis inhibitor (TAFI) by the thrombin/ thrombomodulin complex results in attenuation of fibrinolysis. A latent carboxypeptidase B-like enzyme activated by TAFI cleaves off carboxy-terminal lysine residues from fibrin thereby removing plasmin-binding sites [3].

It is because of the pivotal role of thrombin in coagulation cascade that most strategies for the prophylaxis or treatment of thromboembolic diseases are aimed to block thrombin generation or inhibit its activity.

Earlier we demonstrated that dipeptide Tos-Phe-Arg-OCH_3 was both a substrate and an inhibitor of thrombin ($IC50 = 20\ \mu M$) and it had appropriate characteristics to be transformed into the retro-D-sequence with efficient binding in the active site of thrombin [4]. We have successfully used retro-D-sequence of this dipeptide, D-Arg-D-Phe, as a ligand of the affinity sorbent for obtaining highly purified thrombin with activity of 6000 NIH units/mg [5]. Therefore, in this study we employed the D-Arg-D-Phe motif to create new thrombin inhibitors resistant to enzymatic degradation. Previously, in order to intensify the biologic effects of the inhibitors we modified retro-D peptides by natural compounds [6] and showed that a fatty acid moiety in the thrombin inhibitor structure was a promising scaffold for future development. This approach led us to the study of liporetro-D-peptide affinity for thrombin and selectivity against similar serine proteases.

Abbreviations: DCC, N N'dicyclohexylcarbodiimide; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; HRMS, high resolution mass spectra; LDI, laser desorption ionization; PEG, polyethylene glycol; TAFI, thrombin activatable fibrinolysis inhibitor; TLC, thin layer chromatography.

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We have previously described synthesis and study of peptide-based inhibitors resistant to enzymatic degradation, specifically Chrom-D-Arg-D-Phe-OMe and Laur-D-Arg-D-Phe-OMe, where Chrom is a residue of 3-[6-ethyl-7-hydroxy-3-(4-methyl-thiazol-2-yl)-4-oxo-4H-chromen-2-yl]-propionic acid and Laur is a residue of lauric acid [6]. The comparative inhibitory analysis of the above compounds in relation to thrombin has demonstrated that the modification with the fatty acid residue appeared to be the most successful one. Introduction of the lauric acid residue maximally increased the inhibition effect. Herein we have continued our research and developed the following novel inhibitors: Myr-D-Arg-D-Phe-OMe, where Myr is the residue of myristic acid, and Fmoc-D-Arg-D-Phe-OMe, where Fmoc is a 9-fluorenylmethoxycarbonyl group. We have investigated and compared their inhibitory effect on thrombin, factor Xa, plasmin and trypsin.

In this study, we have synthesized novel thrombin inhibitors, retro-D-peptides modified by fatty acid moieties, which are highly-resistant to enzymatic digestion, investigated their antithrombotic activity and investigated their inhibitory activity and selectivity toward thrombin in comparison to its homologous enzymes. This study provides new insights into the role of the fatty acid tail in the interaction of the peptides with the secondary binding site of thrombin and underscores the importance of future development of new non-toxic and highly selective anticoagulants, resistant to enzymatic degradation.

Materials and Methods

Reagents and equipment

Amino acids, fatty acids, solvents, plates with Silica gel 60 F254 and bovine TPCK-trypsin (40 U/mg) were purchased from Merck (Germany). Bovine factor Xa, human plasmin and condensing agents were obtained from Sigma (Germany). Bovine fibrinogen and polyethylene glycol (PEG) 6000 were purchased from Serva (Switzerland). Chromogenic substrates Chromozym TH (Tos-Gly-Pro-Arg-pNA), for thrombin and trypsin; **S**-2765 (N-Boc-D-Arg-Gly-Arg-pNA), for factor Xa; and S-2251 (H-D Val-Leu-Lys-pNA), for plasmin were obtained from Chromogenix (Sweden). Laser desorption ionization (LDI) high resolution mass spectra (HRMS) were acquired on AUTOFLEX II LRF20 (Bruker Daltonics, Germany) instrument. Enzymatic assays were performed using VMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Thin layer chromatography

Reaction control and purity of the products were monitored by thin layer chromatography (TLC) on plates with Silica gel 60 F254 using the following solvent systems: n-butanol/acetic acid/water, 4:1:1, v/v (A); benzene/ethyl acetate, 5:4, v/v (B); and chloroform/methanol, 8:2, v/v (C). After the chromatography, plates were treated with ninhydrin, iodine, Sakaguchi reagent or ammonium molybdate to reveal the spots.

Chemical synthesis of peptide inhibitors

Compound 1

Fmoc-D-Arg-D-Phe-OMe *(Methyl ester of 9-fluorenylmethoxycarbonyl-D-arginyl-D-phenylalanine).* 9-Fluorenylmethoxycarbonyl-D-arginine 0,77 g (1,94 mmol) and monohydrochloride of D-Phe-OMe (0,42 g, 1,94 mmol) were dissolved in 20 ml of distilled pyridine. The mixture was stirred for 30 min and cooled to 0 °C. N N'dicyclohexylcarbodiimide (DCC) (0,40 g, 1,94 mmol) was added to the cold solution. The reaction mixture was stirred for 6 hours at 0 °C and then left at room temperature for 24 hours. The obtained precipitate was filtered, and the solvent was evaporated under vacuum at 40 °C. The residue was dissolved in butanol-2 and washed with the saturated solution of NaCl and 1 N HCl. Butanol-2 was evaporated, and the residue was crystallized from the ethyl acetate. The yield of the compound was 0,7 g (61%); mp 105-107 °C; Rf = 0,40 in the TLC solvent system A. Data of the element analysis: Calcd. for $C_{31}H_{36}ClN_5O_5$ HCl, %: C, 62,67; H, 6,11; N, 11,79; Found %: C, 63,01; H, 6,09; N, 12,02. LDI HRMS, m/z: Calcd. for $C_{31}H_{35}N_5O_5$: 557,64; Found 580,0587 $(M+Na)^+$. Solubility: >200 mmol/L in 3% dimethyl sulfoxide (DMSO); >10 mmol/L in Tris–HCl buffer (pH 8,0).

Compound 2

Laur-D-Arg-D-Phe-OMe **(***Methyl ester of Nα - lauroyl-D-arginyl-D-phenylalanine).* The compound was prepared according to the method described in [6] where data of element analysis and LDI HRMS are presented. Solubility: >200 mmol/L in H_2O; >50 mmol/L in Tris–HCl buffer (pH 8,0).

Compound 3

Myr-D-Arg-D-Phe-OMe *(Methyl ester of N^{α}- myristoyl-D-arginyl-D-phenylalanine).* A mixture of monohydrochloride Myr-D-Arg (1,1 g, 2,6 mmol) and monohydrochloride of D-Phe-OMe (0,57 g, 2,6 mmol) was dissolved in 20 ml of dimethyl formamide (DMF) and N-methyl-morpholine (0,3 ml, 2,6 mmol) was added. The mixture was stirred for 30 min, cooled to 0 °C, and DCC (0,55 g, 2,6 mmol) was added. The reaction mixture was stirred for 6 hours at 0 °C and then left at room temperature for 24 hours. The obtained precipitate was filtered and the solvent was evaporated under vacuum at 40 °C. The residue was dissolved in butanol-2 and washed with the saturated solution of NaCl and 1 N HCl. Butanol-2 was evaporated, and the residue was crystallized from the mixture of methanol - ethyl acetate. The yield of the compound was 1,4 g (94%); mp 96-98 °C; Rf = 0,43 in the system A. Data of the element analysis: Calcd. for $C_{30}H_{52}ClN_5O_4$, %: C, 61,89; H, 9,00; N, 12,03; Found, %: C, 61,89; H, 9,00; N, 12,03.; LDI HRMS, m/z: Calcd. for $C_{30}H_{52}N_5O_4$: 545,76; Found 568,1890 $(M+Na)^+$. Solubility: >60 mmol/L in H_2O; >10 mmol/L in Tris–HCl buffer (pH 8,0).

Thrombin preparation

Human α-thrombin was isolated from plasma by the method of Fenton et al. [7] with average activity of 3000 NIH units/mg. Thrombin clotting activity was determined as described previously [8].

Enzymatic assays

Inhibition of thrombin clotting activity (IC50 determination)

The amount of thrombin in the assay was chosen for the 0,1% solution of fibrinogen (90% purity) to clot within 15–20 sec under the described conditions [8]. Inhibition of thrombin clotting activity by synthesized peptide inhibitors was studied under the following conditions. The inhibitor was dissolved in the assay buffer consisting of 0,04 M Tris–HCl buffer (pH 7,3), 0,15 M NaCl and 0,66% PEG 6000 and was added to an equal volume (0,5 ml) of 0,2% fibrinogen solution in the same buffer. The reaction mixture was incubated at 29 °C for 5 min, then 20 μl of thrombin solution (1 NIH unit) was added and the clot formation time was recorded manually using a timer. The clotting time was determined three times for 5–6 various concentrations of peptides. The value of IC50 was calculated from the curve where $(t-t_0)/t_0$ was plotted versus [I], where t_0 - clotting time in the absence of the inhibitor; t - clotting time in the presence of the inhibitor; and [I] - concentration of the inhibitor.

Inhibition of thrombin, trypsin, factor Xa and plasmin amidolytic activities

The inhibition constant *Ki* for all four enzymes was determined assaying the effects of different concentrations of the inhibitors on