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Cloning, purification and biochemical characterization of dipetarudin, a new chimeric thrombin inhibitor

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Abstract

The development of thrombin inhibitors could provide invaluable progress for antithrombotic therapy. In this paper, we report the cloning, purification and biochemical characterization of dipetarudin, a chimeric thrombin inhibitor composed of the N-terminal head structure of dipetalogastin II, the strongest inhibitor from the assassin bug *Dipetalogaster maximus*, and the exosite 1 blocking segment of hirudin, connected through a five glycine linker. The cloning of dipetarudin was performed by a simple method which had not been used previously to clone chimeras. Biochemical characterization of dipetarudin revealed that it is a slow, tight-binding inhibitor with a molecular mass (M_r=7560) and a thrombin inhibitory activity (K_i=446 f*M*) comparable to r-hirudin.

Keywords: Cloning; Purification; Dipetarudin; Thrombin inhibitor

1. Introduction

Thrombin is the final serine protease in the activation of the blood coagulation network. It initiates blood clotting by cleaving soluble fibrinogen and also activates other procoagulant enzymes including factors V, VIII, XI and XIII, and the anticoagulant enzyme protein C [1–3]. Thrombin also has marked effects on a variety of cells including platelets, endothelial and smooth muscle cells, which seem to be transmitted via specific thrombin receptors called protease-activated receptors (PARs) [4]. Given its diverse functions, the development of thrombin inhibitors could be a new alternative in the treatment of patients with thromboembolic disorders.

Haematophagous animals have evolved highly specific strategies to inhibit the coagulation system of their victims, among which the thrombin inhibitors are the most prominent. Hirudin is the first and intensively studied thrombin inhibitor from blood sucking animals. It was isolated from the medicinal leech *Hirudo medicinalis* [5] and characterized as a specific slow, tight-binding thrombin inhibitor with a K_i value of 21 f*M* and a relative molecular mass (M_r) of ~7000 [6], that blocks the access of substrates to the active site of thrombin with its disulfide-linked N-terminal core and occupies the anion binding exosite 1 (ABE 1) with its carboxy terminal tail. Recombinant hirudin (r-hirudin) is not sulfated at Tyr_{63} and exhibits a tenfold reduced affinity for thrombin (K_i=217 f*M*) [7,8].

Another thrombin inhibitor, dipetalogastin II, was

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isolated from the assassin bug *Dipetalogaster maximus* [9] and cloned from a stomach library of this insect [10]. Recombinant dipetalogastin II (rDII) is also a bifunctional thrombin inhibitor interacting with the active site and the ABE 1 of thrombin. Kinetic analysis of rDII demonstrated a thrombin inhibitory activity stronger than r-hirudin (K_i=49.3 f*M*), but in contrast to hirudin, rDII is much larger (M_r=12 909).

In order to decrease the antigenic potential and to improve the therapeutic profile of dipetalogastin II, we generated a smaller chimeric thrombin inhibitor by replacing the bulky second head structure of dipetalogastin II by the ABE 1 blocking segment of hirudin. Moreover, knowing that the distance between the active site and the ABE 1 blocking segment is very important to ensure their interaction with thrombin, a flexible linker of five glycine residues between both blocking segments was inserted. The new chimeric thrombin inhibitor was designated dipetarudin. In this paper, we describe its cloning, purification and biochemical characterization.

2. Experimental procedures

2.1. Materials

Human α-thrombin (EC 3.4.21.5) was supplied by Kordia Laboratory Supplies, Leiden, The Netherlands. Trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), plasmin (EC 3.4.21.7) and activated factor X (EC 3.4.21.6) were from Sigma–Aldrich, Steinheim, Germany. The chromogenic substrates H-D-Phe-Pip-Arg-*p*-nitroaniline (S2238), N-α-Z-D-Arg-Gly-Arg-*p*-nitroaniline (S2765), H-D-Val-Leu-Lys-*p*-nitroaniline (S2251) and MeO-Suc-Arg-Pro-Tyr-*p*-nitroaniline (S2586) were purchased from Chromogenix, Mölndal, Sweden. Ecarin and H-D-CHG-Ala-Arg-*p*-nitroaniline (Pefachrome TH) were purchased from Pentapharm, Basel, Switzerland. The *Escherichia coli* strain JM 105, the expression vector pGEX-5X-1 and the primer EXP1 were purchased from Amersham Pharmacia Biotech Europe, Freiburg, Germany. The primer DIPCHI-R2 was purchased from Carl Roth, Karlsruhe, Germany. Pooled human plasma obtained by plasmapheresis of 12 healthy donors was purchased from the Red Cross Blood Donor Service, Gera, Germany.

2.2. Cloning of dipetarudin

The dipetalogastin cDNA inserted into the plasmid pV/6 [10] was linearized with Hind III, and used as template in the PCR (ten cycles of 1 min at 94 °C, 1 min 30 s at 60 °C, and 2 min 30 s at 72 °C). The primer EXP 1 was used as a sense primer, and degenerate DIPCHI-R2 as antisense primer. The primers were designed according to the N-terminal and internal sequence of dipetalogastin II and C-terminal sequence of hirudin and incorporated sequences at their 5′ end for creation of restriction sites for Eco RI and Not I.

EXP1: 5′ -CTC GAA TTC CAG GGG AAT CCT TGT-3′

DIPCHI-R2: 5′ -CTC GCG GCC GC TTG CAA GTA TTC TTC TGG AAT TTC TTC GAA GTC TCC TCC TCC TCC TCC GCA TGG GCC TTC/T A-3′

The PCR products were purified by electrophoresis on a 1% agarose gel using the QIAEX II gel extraction kit according to the manufacturer's guidelines (Qiagen), digested with Eco RI and Not I, subcloned into the expression vector pGEX-5X-1 which contains the glutathione S-transferase (GST) gene, using T4 DNA ligase and transformed in *E. coli* JM 105.

To identify bacterial colonies that contained the dipetarudin sequence, the plasmid DNA from transformed bacteria was isolated using the Midiprep System from Qiagen and examined by DNA sequencing [11].

2.3. Expression and purification of dipetarudin

Expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.76 m*M* and the purification of the expressed fusion protein was accomplished using the glutathione S-transferase (GST) Gene Fusion System according to the manufacturer's guidelines (Amersham Pharmacia Biotech). GST fusion protein was purified directly from bacterial lysates using the affinity matrix glutathione Sepharose™ 4B. Reduced glutathione (10 m*M*) in 50 m*M* Tris–HCl (pH 8.0) was used for

elution of the fusion protein from the affinity matrix. Cleavage of the recombinant dipetarudin from GST was achieved using factor Xa (10 U activated factor X (FXa)/mg fusion protein) whose recognition sequence is located immediately upstream from the multiple cloning site. Finally, dipetarudin was purified by reversed-phase HPLC (RP-HPLC) using a Vydac™ C_{18} column (25.0×2.2 cm) with a linear gradient that changed in 122 min from 5% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) to 60% acetonitrile in water with 0.1% TFA on a Shimadzu LC-8A HPLC system (Shimadzu, Germany).

2.4. Analysis of anticoagulant activity

The ecarin clotting time (ECT) [12] was performed in a CL4 coagulometer (Behnk-Elektronik, Germany) at 37 °C. A 80-μl aliquot of inhibitor solution in 0.05 *M* Tris–HCl buffer (pH 7.5) containing 0.154 *M* NaCl was added to 200 μl plasma and preincubated for 3 min at 37 °C. The reaction was started by addition of 20 μl ecarin solution (5 EU/ml in 0.154 *M* NaCl/0.05 *M* $CaCl_2$) and the time until clotting was measured. The final concentration of inhibitor that produced a prolongation of the clotting time to 100 s (CT_{100}) was calculated from the concentration–clotting time curve.

2.5. Specificity of serine protease inhibition

Analysis of specificity of serine protease inhibition was performed as described previously [10]. Briefly, different concentrations of inhibitor and the enzyme (at a constant concentration) were incubated in 50 m*M* Tris–HCl buffer (pH 8.0) containing 0.1 *M* NaCl and 0.1% PEG 6000 for 5 min at 37 °C. The reaction was started by addition of a chromogenic substrate solution diluted in the same buffer. Formation of *p*-nitroaniline was recorded over 10 min at 405 nm and the initial rate of the reaction was determined. Enzyme activities in percentage were calculated from calibration curves.

Solutions of S-2238 (0.1 m*M*), S-2765 (0.5 m*M*), S-2251 (1 m*M*), Pefachrome TH (0.1 m*M*), and S-2586 (0.5 m*M*) were used as chromogenic substrate for thrombin, FXa, plasmin, trypsin and α-chymotrypsin, respectively. The final concentrations of the enzymes were 0.0775 n*M* for thrombin, 0.36 n*M* for FXa, 1.3 n*M* for plasmin, 0.5 n*M* for trypsin and 0.125 n*M* for α-chymotrypsin.

2.6. Determination of the dissociation constant K_i

The dissociation constant (K_i) of dipetarudin was determined according to Stone and Hofsteenge [6]. The assay was performed in 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.1 *M* NaCl and 0.1% PEG 6000, dipetarudin (at different concentrations) and S-2238 (final concentration: 30 μ*M*) in a final volume of 950 μl. The reaction was started by addition of 50 μl of α-thrombin (final concentration: 77.5 p*M*). The progress curve of formation of *p*-nitroaniline was recorded spectrophotometrically at 405 nm over 10 min on a M400 spectrophotometer (Carl Zeiss Jena, Germany). The dissociation constant (K_i) was determined by non-linear regression analysis according to the theory of slow, tight binding or slow binding inhibition [13].

The progress curves obtained for several concentrations of dipetarudin were fitted to Eq. (1) by non-linear least-squares,

$$[P] = v_s t + ((v_0 - v_s)(1 - \gamma)/\gamma k_{obs}) \times \ln((1 - \gamma e^{-kobst})/(1 - \gamma)) + d \quad (1)$$

The symbols v_0, v_s and k_{obs} represent the velocity observed in the absence of the inhibitor (initial velocity), the steady-state velocity and the apparent first-order rate constant, respectively. P is the amount of product formed at time "t", d is a displacement term to account for the fact that at $t=0$ the absorbance may not be accurately known. γ is a factor related to K_i, total enzyme concentration (E_t) and total inhibitor concentration (I_t) by the equation:

$$\gamma = (K_i' + E_t + I_t - Q)/(K_i' + E_t + I_t + Q)$$

and

$$Q = ((K_i' + E_t + I_t)^2 - 4E_tI_t)^{1/2}$$

The values of v_s obtained by Eq. (1) were then incorporated to Eq. (2) [6] to obtain apparent dissociation constants (K_i'):

$$v_s = (v_0/2E_t)[[(K_i' + xI_x - E_t)^2 + 4K_i'E_t]^{1/2} - (K_i' + xI_x - E_t)] \quad (2)$$

K_i' was related to the dissociation constant of the inhibitor (K_i) by the equation:

$$K_i' = K_i(1 + S/K_m) \quad (3)$$

2.7. Molecular mass determination and N-terminal sequence analysis

The molecular mass was determined by matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) analysis using a Kompact Probe (Kratos Analytical, UK). The matrix was prepared as a stock solution containing 10 mg α-cyano-4-hydroxy-cinnamic acid in 1 ml 50% acetonitrile/50% water containing 0.1% TFA. Bovine insulin (M_r = 5733.5), bovine cytochrome *c* (M_r = 12 233) and angiotensin II (M_r = 1046) were used as samples for mass calibration. N-terminal sequence analysis was performed as described previously [10].

2.8. Protein concentrations

The fusion protein concentration was routinely determined according to Bradford [14], or with the Bicinchoninic acid (BCA) protein assay [15] according to the Sigma procedure TPRO-562, using BSA as standard. The concentration of dipetarudin was determined by active site titration according to Dang and Di Cera [16] in a M400 spectrophotometer (Carl Zeiss Jena, Germany).

3. Results

A chimeric thrombin inhibitor containing the first 48 amino acids of dipetalogastin II and the hirudin-derived C-terminal peptide DFEEIPEEYLQ that binds to the ABE 1 of thrombin was cloned and designated dipetarudin.

3.1. Production of recombinant dipetarudin

The result of the PCR amplification as checked on agarose gels was a single predominant band of ~0.2 kb (Fig. 1), which was isolated with the QIAEX II gel extraction kit, digested with the restriction enzymes Eco RI and Not I and then subcloned into the expression vector pGEX-5X-1, which was also digested with the same enzymes. This vector allows inducible, intracellular expression of dipetarudin as a fusion with GST. The resulting fusion protein was purified and cleaved as described under experimental procedures. The released dipetarudin was further separated and purified by RP-HPLC (Fig. 2A). The isolated material was homogeneous and pure as judged by SDS–polyacrylamide gel electrophoresis (data not shown) and HPLC analysis (Fig. 2B). Its identity was checked by N-terminal sequence and molecular mass determination.

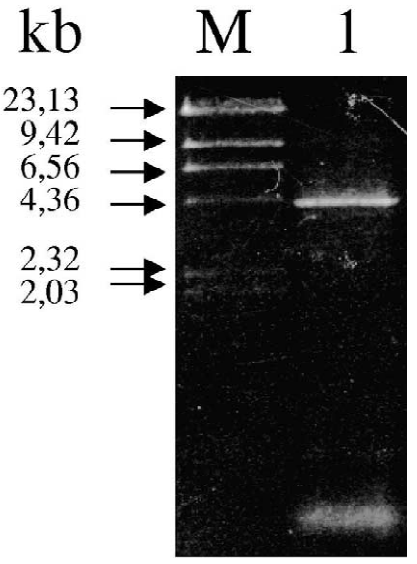

Fig. 1. PCR amplification of the dipetarudin sequence was performed using the EXP 1 and DIPCHI-R2 primers and the dipetalogastin cDNA clone (plasmid pV/6) linearized with Hind III as template (line 1). Lambda DNA digested with Hind III was electrophoresed in lane M as size markers whose length is indicated in the left margin.

N-terminal sequencing verified the correct processing of dipetarudin. Its aminoterminus is enlarged by the peptide GIPE derived from the polylinker of the expression vector. The molecular mass of dipetarudin determined by MALDI-TOF (M_r = 7560.8) is in agreement with that calculated by its amino acid composition (M_r = 7561.3).

3.2. Kinetic of thrombin inhibition

Kinetic analysis demonstrated that dipetarudin acts as a slow, tight binding inhibitor of thrombin. A set

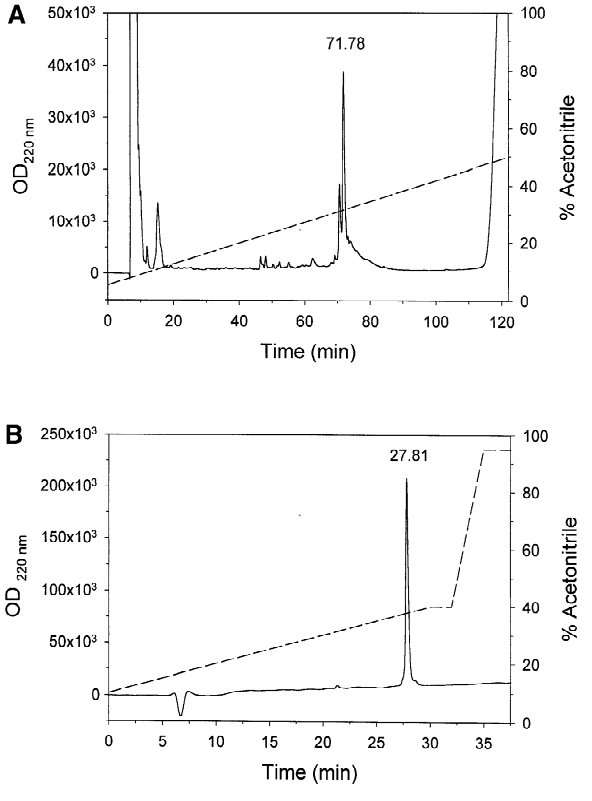

Fig. 2. Reversed phase-high-performance liquid chromatography of dipetarudin. (A) A 10-mg sample of fusion protein digested with FXa was loaded onto a Vydac™ C_{18} column (25×2.2 cm). The elution was performed with a linear gradient of 5–50% acetonitrile in 0.1% TFA in 122 min at a flow-rate of 10 ml/min. (B) The purified dipetarudin was loaded onto a Vydac™ C_{18} column (25×0.5 cm). A gradient of 10–40% acetonitrile in 0.1% TFA was applied over 30 min at a flow-rate of 1 ml/min. The whole procedure was carried out at room temperature. Absorbance was read at 220 nm.

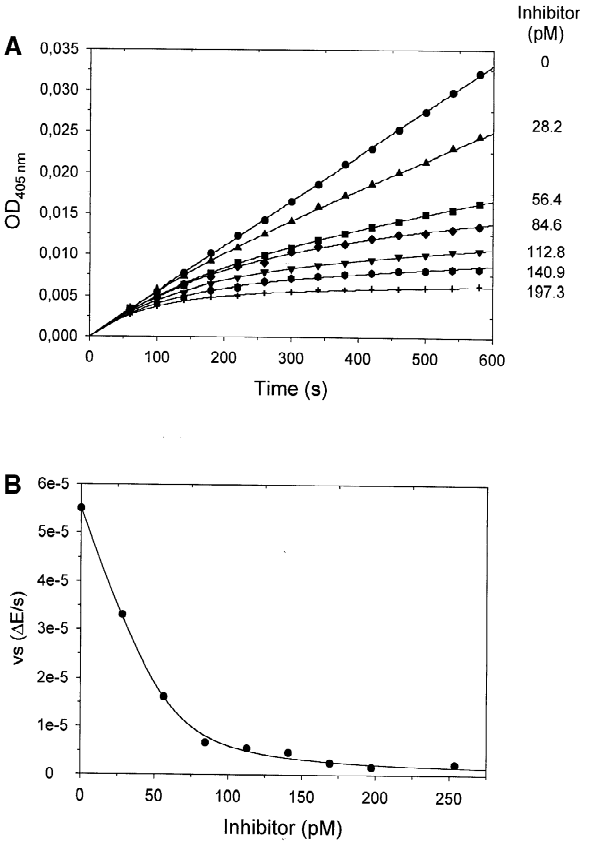

Fig. 3. Slow, tight-binding inhibition of thrombin by dipetarudin (representative data of one to four experiments). (A) Progress curves of the thrombin (77.5 p*M*) catalyzed hydrolysis of the chromogenic substrate S-2238 (30 μ*M*) in the presence of different dipetarudin concentrations in p*M*. The solid lines are the non-linear least-squares fitting of the data using Eq. (1). (B) Dependence of the steady-state velocities obtained from the progress curve analysis of the thrombin (77.5 p*M*) catalyzed hydrolysis of S-2238 (30 μ*M*) in the presence of dipetarudin.

of progress curves was obtained at several dipetarudin concentrations, and the data were fitted by non-linear regression to Eq. (1) (Fig. 3A). For each inhibitor concentration a rapid initial phase that decreased to a slower steady-state velocity was observed. The values of v_s obtained by Eq. (1) were then incorporated into Eq. (2) to obtain apparent dissociation constants (K_i') (Fig. 3B). Finally, the dissociation constant (K_i) was calculated by the equation $K_i' = K_i(1 + S/K_m)$. The K_m value of the chromogenic substrate (S-2238) was 2.15±0.35 μ*M*. The results of these kinetic analyses are shown in

Table 1
Kinetic constants for the interaction of thrombin with dipetarudin

Inhibitor	K_i (M) $\times 10^{-15}$	k_{on} (M^{-1} s^{-1}) $\times 10^{6}$	k_{off} (s^{-1}) $\times 10^{-4}$
r-Hirudin[a]	237±6	137±3.0	0.317±0.011
rDII[a]	49.3±22.3	ND	ND
Dipetarudin	446.1±84.7	842.3±178.16	3.66±0.46

Values of dipetarudin represent the mean±SD of four determinations. ND, not determined.

[a] The values for r-hirudin [8] and rDII [10] have been previously reported.

Table 1. The affinity of dipetarudin for human α-thrombin is nine-fold lower than that of rDII (K_i of 446 vs. 49.3 f*M* for rDII) and about two-fold lower than that of r-hirudin ($K_i = 217$ f*M*).

3.3. Specificity toward serine proteases

When thrombin was incubated with rDII at a molar ratio 1:1, its amidolytic activity could not be detected [10]. At the same conditions, dipetarudin produced 92% of thrombin inhibition (Fig. 4).

Replacement of the second head structure of dipetalogastin II by the hirudin fragment has no effect on the inhibition of trypsin and plasmin amidolytic activities. Thus, at a 110-fold molar excess of rDII or dipetarudin over trypsin, 38 and 33% of inhibition was observed, respectively, while 44 and 48% of inhibition was observed at a 110-fold molar excess over plasmin of rDII or dipetarudin, respectively. Furthermore, no inhibition of α-chymotrypsin or FXa by these inhibitors was detectable even at a 200-fold molar excess of the inhibitor (data not shown).

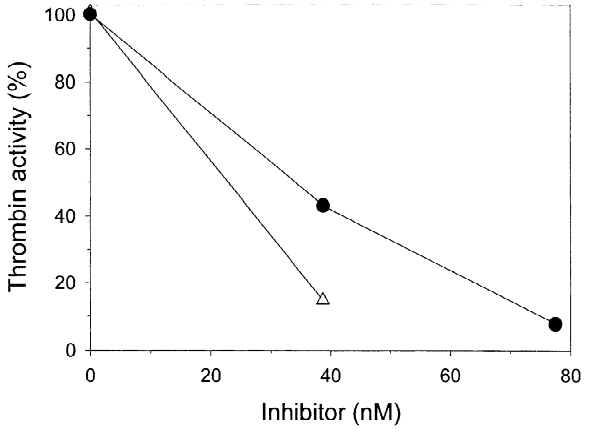

Fig. 4. Inhibition of the amidolytic activity of human α-thrombin by increasing concentrations of rDII (open triangles), and dipetarudin (filled circles). Various amounts of each inhibitor were mixed with a fixed concentration of thrombin (77.5 p*M*) in Tris–HCl buffer (pH 8.0). The mixture was incubated at 37 °C for 5 min. The activity of free thrombin was assayed by adding the chromogenic substrate S-2238 (50 μ*M*) and the increase in absorbance at 405 nm was then measured.

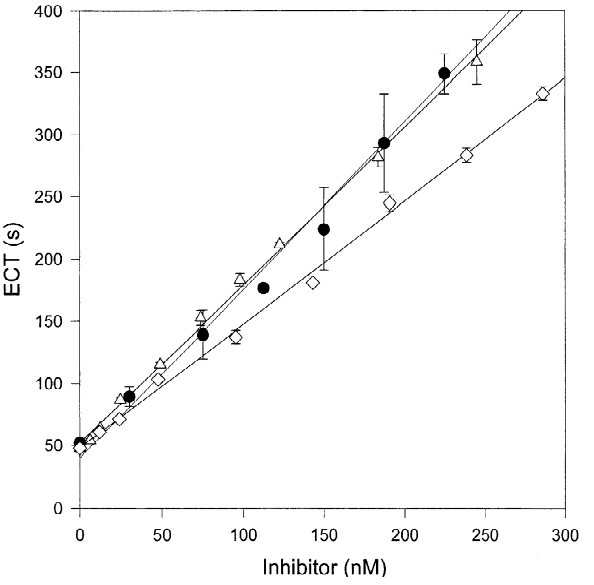

Fig. 5. Influence of increasing concentrations of rDII (open triangles), dipetarudin (filled circles) and hirudin (open diamonds) on the ecarin clotting time. Various amounts of each inhibitor were preincubated with plasma for 3 min at 37 °C. The reaction was started by adding ecarin and then the time until a clot formed was measured. Each point represents a mean±SD of three or more individual determinations. The data for rDII and hirudin have been previously published [10].

3.4. Anticoagulant activities

To study the anticoagulant activity of the dipetarudin in vitro, its influence on the ecarin clotting time (ECT) was investigated. Fig. 5 shows the concentration-dependent inhibitory activities of rDII, hirudin and dipetarudin on the ECT. The anticoagulant activities of all these inhibitors were concentration-dependent. Dipetarudin has similar anticoagulant activity to rDII at equivalent dosage ($CT_{100} = 44.15$ n*M* for dipetarudin vs. 37.51 n*M* for rDII). These inhibitors were slightly more potent in prolonging the ecarin clotting time than hirudin ($CT_{100} = 51.57$ n*M*).

4. Discussion

The current results describe the generation of a chimeric thrombin inhibitor between the N-terminal

domain of dipetalogastin II, the strongest thrombin inhibitor from the blood-sucking insect *Dipetalogaster maximus*, and the C-terminal fragment of hirudin.

For the PCR amplification, we have designed a downstream primer, DIPCHI-R2 whose 5′ end codifies the sequence of the hirudin carboxy terminal tail and five glycine residues. It matches the codons in the dipetalogastin cDNA that codifies the amino acids 44–48. Thus, the end of the PCR-generated DNA fragment incorporated the C-terminal hirudin sequence which was not present in the original template.

It is generally assumed that PCR is acceptably efficient when using primers with 15–20% bp mismatches with the template and that mismatches at a primer's 3′ end cause more serious problems than the same mismatch ratio at the 5′ end because they interfere with extension by the DNA polymerase [17,18]. DIPCHI-R2 has 82% bp mismatches with the template at the 5′ end; however, using this primer we were able to amplify the DNA sequence of a new chimeric thrombin inhibitor. At present, the cloning of chimeras using this method has not been reported in the literature.

In fact, the cloning of a chimeric thrombin inhibitor among the leech-derived tryptase inhibitor and the C-terminal peptide of hirudin by cassette mutagenesis has been reported [19]. This technique involves replacing the wild-type sequence by synthetic double-stranded oligonucleotides also called cassettes. Because the cassettes need different restriction sites at both ends and naturally occurring restriction sites hardly ever fulfil these criteria, it is usually necessary to carry out one or more rounds of site-directed mutagenesis to create restriction sites at the appropriate localization in the wild-type gene and sometimes the introduction of these sites changes the amino acid sequence encoded by the gene, and therefore it is necessary to determine whether this modification alters the function of the wild-type protein.

Therefore, cassette mutagenesis is a more laborious procedure for adding the C-terminal hirudin sequence to an active site blocking segment of thrombin than the technique applied in this study. One limitation in the application of this technique to generate hybrid molecules is that the changes can only be made at the end of a PCR product.

On the other hand, the interaction of dipetarudin with α-thrombin displayed slow-binding kinetics, evidence of which was the downward concavity displayed for the progress curve when α-thrombin was added to the reaction medium containing inhibitor and the substrate S-2238. In addition to exhibiting slow-binding kinetics, dipetarudin significantly inhibited α-thrombin at a concentration similar to that of the enzyme, indicating that it is a slow, tight-binding inhibitor [20,21].

In accordance with the kinetic data, the potency of dipetarudin to inhibit the thrombin amidolytic activity was lower than that of rDII. However, the anticoagulant activity of dipetarudin is not in agreement with the kinetic data. Thus, although dipetarudin has a nine-fold lower K_i than rDII and a two-fold lower K_i than r-hirudin, its anticoagulant activity in clotting assays is similar to rDII and slightly better than r-hirudin. This finding seems to suggest that this chimera interacts better with meizothrombin than with α-thrombin as the ECT method used for quantitative determination of thrombin inhibitors is based on the interaction of these drugs with meizothrombin, the intermediate product of ecarin-mediated prothrombin–thrombin conversion [12].

Like rDII, dipetarudin was able to inhibit trypsin and plasmin, but not chymotrypsin and FXa at a large molar excess over these enzymes. Other modifications in the dipetarudin's structure are necessary to improve its specificity toward thrombin.

In summary, dipetarudin is a genetically engineered potent thrombin inhibitor with a molecular mass and an inhibitory activity comparable to hirudin. It might have therapeutic potential as an antithrombotic agent or anticoagulant (e.g. in haemodialysis and during extracorporeal circulation), but its suitability for possible in vivo application will depend on its toxicity, antigenicity, bioavailability and other pharmacokinetic parameters. Such investigations are in progress.

5. Nomenclature

ABE 1	anion binding exosite
ECT	ecarin clotting time
FXa	activated factor X
GST	glutathione S-transferase
IPTG	isopropyl-β-D-thiogalactoside

rDII	recombinant dipetalogastin II
RP-HPLC	reversed-phase high-performance liquid chromatography
TFA	trifluoroacetic acid

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