Triabin, a Highly Potent Exosite Inhibitor of Thrombin*

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Christiane Noeske-Jungblut‡§, Bernard Haendler‡, Peter Donner‡, Alejandro Alagon¶, Lourival Possani¶, and Wolf-Dieter Schleuning‡

From the Research Laboratories of Schering AG, D-13342 Berlin, Germany and the Universidad Nacional Autonoma de Mexico, Instituto de Biotecnologia, Cuernavaca, Morelos 62271, Mexico

Triabin, a new thrombin inhibitor, has been purified from the saliva of Triatoma pallidipennis, a blood-sucking triatomine bug. It forms a noncovalent complex with thrombin at a molar ratio of 1:1, inhibits thrombin-induced platelet aggregation, and prolongs thrombin clotting time and activated partial thromboplastin time. However, it only minimally suppresses the amidolytic activity of thrombin, as measured by a chromogenic peptide substrate assay. It completely blocks trypsin-catalyzed cleavage of thrombin, probably via protection of the anion-binding exosite and inhibits the effect of thrombomodulin on thrombin in a dose-dependent fashion. These results indicate that the inhibitor is directed toward the anion-binding exosite of thrombin. The protein was partially sequenced and the information used to isolate cDNA clones from a T. pallidipennis salivary gland library. Four slightly polymorphic variants coding for mature proteins of 142 amino acids preceded by a putative leader sequence were obtained. The recombinant protein expressed in the periplasmic space of Escherichia coli has a biological activity similar to that of salivary triabin, as tested in a thrombin-induced platelet aggregation assay. In addition, recombinant triabin inhibits thrombin-catalyzed hydrolysis of fibrinogen with a $K_i$ of about 3 pM.

Evolution has provided hematophageous animals with specific mechanisms to interfere with blood coagulation. Factor $Xa$ inhibitors were isolated from the leech Haementeria officinalis (1) and the tick Ornithodoros moubata (2). Likewise, several proteins which inhibit thrombin have been described. The most widely known is hirudin, which was identified almost 40 years ago in the leech Hirudo medicinalis (3). Thrombin is a key enzyme in the blood coagulation cascade and a strong inducer of platelet aggregation. Three functional domains of thrombin have been described: an active site, a heparin-binding site, and a so-called anion-binding exosite mediating the binding to fibrinogen (4), the platelet receptor (5), and thrombomodulin (6).

The triatomine bug Rhodnius prolixus has been reported to contain a Kazal-type inhibitor which seems to be specifically directed to the active site of thrombin (7). We investigated the related triatomine bug Triatoma pallidipennis, which also belongs to the family of Reduviidae or assassin bugs, and recently described the purification, clotting, and expression of a protein called pallidipin which specifically inhibits collagen-induced platelet aggregation (8). Now we have identified a thrombin inhibitor in the saliva of this insect and report its purification and characterization, and the cloning and expression of the corresponding cDNA. In contrast to the inhibitor from R. prolixus, the protein we isolated from T. pallidipennis and called triabin appears to be directed at the anion-binding exosite of thrombin.

EXPERIMENTAL PROCEDURES

Collection of Saliva—The saliva was obtained from T. pallidipennis bugs reared in captivity. Adult insects were hand stimulated to eject saliva which was collected in siliconized capillary tubes and lyophilized.

Purification of Triabin—The saliva was applied onto a Superose 12 column (Pharmacia Biotech Inc.) equilibrated in 10 mM Tris-HCl, pH 7.4, 0.2 mM CHAPS. The fractions with inhibitory activity were pooled and loaded onto a thrombin-Sepharose 4B column prepared by coupling thrombin to CH-activated Sepharose as described by the manufacturer (Pharmacia). The column was washed with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl$_2$, 3.3 mM Na$_2$HPO$_4$, 5.5 mM glucose, 3.8 mM Hepes, pH 7.4) followed by 10 mM sodium acetate, pH 4.5. The inhibitor was eluted with 10 mM glycine, pH 2.5. The fractions were titrated to pH 7 and pooled. The purity of the preparation was determined by SDSPolyacrylamide gel electrophoresis according to Laemmli (9).

Lys-C Cleavage—45 µg of triabin were incubated with 1 µg of Lys-C (Boehringer Mannheim) for 6 h at 37 °C. The generated peptides were separated by reversed phase high performance liquid chromatography using a Superspher RP column (MZ-Analysentechnik) with a gradient from 0.1% trifluoroacetic acid in water to 0.08% trifluoroacetic acid in 70% acetonitrile. Selected peaks were subjected to amino acid sequencing.

Protein Sequence Analysis—Triabin, modified with vinylpyridine (10), was subjected to automated Edman degradation in a gas-phase sequenator as described by the manufacturer (Applied Biosystems Inc.).

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis and native electrophoresis in the absence of detergent was performed according to Laemmli (9) and Maurer (11), respectively. Proteins were stained with Coomassie Brilliant Blue.

Determination of Protein Concentration—The method of Bradford (12) was used to determine protein concentration.

Partial Proteolysis of Thrombin—60 pmol of thrombin (Sigma) were preincubated either with 70 pmol of triabin or buffer (100 mM Tris-HCl, pH 7.8, 150 mM NaCl) for 5 min at 37 °C followed by the addition of 40 ng of trypsin. After 1 h incubation at 37 °C the reaction was stopped with 0.5 µg of Pefabloc (E. Merck). The samples were boiled in sample buffer and electrophoresed according to Laemmli (9).

Activity Assays—Fibrinogen clotting activity was measured as described previously (13). This assay was used for the identification of active fractions during the purification procedure.

Aggregation of washed platelets was measured as described earlier (8). Briefly, thrombin (human from Sigma, 1 mU) and triabin or hirudin preincubated for 1 min at 37 °C were added to washed platelets and aggregation was monitored in an aggregometer (Micron).

To assay amidolytic activity, 20 µl of thrombin (human from Sigma, 45 mU final concentration) were incubated with 80 µl of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 100 µl of inhibitor or buffer for 2 min at 37 °C

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X80246.

§ To whom correspondence should be addressed. Tel.: 49-30-468-1596; Fax: 49-30-4691-6707.
in microtiter plates followed by the addition of 100 μl of H-D-Phe-Pip-Arg-p-nitroaniline (S2238, Kabi Vitrum, 0.5 mM). The absorbance at 405 nm was recorded for 60 min in a microplate reader and the initial rate of p-nitroaniline liberation determined.

For thrombin clotting time assays, 100 μl of thrombin (Behring, 3 IU/ml) and 100 μl of inhibitor or diethylbarbiturate acid buffer (Behring) were incubated for 1 min at 37°C. After addition of 100 μl of polyclonal (control plasma from Behring), the time until a clot formed was measured. The same method was used to determine reptilase activity but 200 μl of reptilase (Sigma, 5 μg/ml) was added instead of 100 μl of thrombin.

To determine activated partial thromboplastin time (APTT), 100 μl of pooled human plasma, 10 μl of inhibitor, and 100 μl of APTT reagent (Pathromtin, Behring, Germany) were incubated for 3 min at 37°C. After addition of 100 μl of prewarmed 25 mM CaCl₂, the time until a clot formed was measured in a fibrometer (Starsettd Biomatic B10).

To measure factor Xa and trypsin activities, triabin was incubated with factor Xa (American Diagnostica Inc.) or with trypsin (up to a molar ratio of 43:1 for 2 min at 37°C) followed by the addition of N-benzoyl-1-leu-glu-arg-p-nitroaniline (S2222, Kabi Vitrum, final concentration 0.167 mM). After 20 min at 37°C, the absorbance at 405 nm was measured and compared to the control value without triabin.

Protein C activation was measured in 96-well plates in an assay diluted (20 μl Tris-HCl, pH 7.4, 0.1 mM NaCl, 0.1% bovine serum albumin, 2.5 mM CaCl₂) as described previously. Triabin, thrombomodulin (35 ng/ml, kindly provided by Dr. John Morser, Berlex Biosciences), thrombin (Sigma, 0.44 units/ml), and protein C (Genzyme, 3 μM) were incubated for 1 h at 37°C. The reaction was quenched with hirudin (American Diagnostica Inc., 0.16 units/ml) and 100 μl of vinyl-L-leu-Arg-p-nitroaniline (S2266, Kabi Vitrum, 1 mM) were added. The absorption at 405 nm was recorded for 15 min in a microplate reader.

Cloning of Triabin cDNA—A tag was first generated by polymerase chain reaction using T. pallidipennis salivary gland cDNA (8) as template. Sense (5′-GCGAATAGCTAGCAGAAGGTGACGAC-3′) and antisense (5′-TTRTTRTANCCRATYTCNACRATYTGNGT-RAA-3′) primers deduced from the N-terminus and an internal region of the salivary gland cDNA were used in 40 cycles of 2 min denaturation at 94°C, 1 min 30 s annealing at 52°C, and 2 min elongation at 72°C. The amplified 200-base pair fragment was subcloned and sequenced using the dyeideoxycase chain termination method (15). A screening probe was derived from it using the Taq polymerase (Perkin Elmer) in 5 cycles of 2 min 30 s at 94°C, 2 min at 40°C, and 3 min 30 s at 72°C, in the presence of labeled dCTP (Amershram) and using the antisense primer. A T. pallidipennis DNA library (8) was screened after adding peroxidase-labeled antibodies (8C2–5) against fibrinopeptide A and applied to a microtiter plate coated with antigen. After 30 min incubation, peroxidase activity was determined using o-phenylenediamineH₂O₂ as substrate. The velocities of fibrinogen cleavage were determined from the gradient of fibrinopeptide A concentration between 0 and 4 min. The Kᵢ was determined by nonlinear regression analysis of velocity versus substrate concentration according to Duggleby (20) using the computer program k-cat (Europa Scientific Software Corp.). The Kᵢ was graphically determined by the method of Dixon (21).

RESULTS

The saliva of T. pallidipennis was found to inhibit not only platelet aggregation induced by collagen but also aggregation mediated by thrombin. These two inhibitory activities were separated by gel filtration, although the two proteins have similar molecular mass. The saliva was incubated on a 12 column with a buffer containing only low salt concentrations which led to a retardation of pallidipin but not of the thrombin inhibitor. The thrombin inhibitor was further purified by affinity chromatography on thrombin Sepharose. 11 μg of purified inhibitor, which we named triabin, were recovered from 10 mg of dried saliva. The purity of this preparation was ascertained by SDS-polyacrylamide electrophoresis, which showed a single band with an apparent molecular mass of 21 kDa under reducing conditions and 18 kDa under nonreducing conditions (Fig. 1, lanes 7 and 8).

In order to investigate the triabin-thrombin complex, we incubated triabin with thrombin at different molar ratios and analyzed the probes by nondenaturing polyacrylamide gel electrophoresis. As shown in Fig. 1, the inhibitor was totally bound in a complex with thrombin when the molar ratio of triabin to thrombin was 1:1, whereas free triabin was detected at a ratio of 1.5:1 (lanes 3 and 4). This complex was not covalently linked since it could be separated by SDS-polyacrylamide gel electrophoresis (lane 6).

Triabin inhibited thrombin-induced platelet aggregation in a dose-dependent fashion (Fig. 2). If platelet aggregation was induced by 1 nM thrombin, total inhibition was observed at 1 μM.
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Triabin. The dose-response curve was almost identical to that of hirudin. In contrast, the COOH-terminal tail of hirudin, hir(54–65), also called hirugen, exhibited a nearly 500 times lower activity (Fig. 2). No effect of triabin was noted on the aggregation induced by collagen or ADP (not shown). Triabin inhibited not only platelet aggregation but also interfered with the other known activities of thrombin. A 4-fold prolongation of thrombin-clotting time was measured with 22 nM triabin (Fig. 3). Clotting of plasma can also be catalyzed by reptilase, an enzyme from the snake venom of Bothrops atrox (22). Triabin did not inhibit this enzyme (data not shown). Activated partial thromboplastin time was prolonged 4-fold by about 600 nM triabin and about 400 nM hirudin. While hirudin already showed an effect at 10 nM, triabin was only active at concentrations higher than 500 nM (Fig. 3). In contrast to its strong inhibitory effect in the assays described above, triabin hardly affected thrombin activity in an amidolytic assay using a synthetic peptide substrate (Fig. 4). Only a 35\% inhibition was achieved with a 3-fold molar excess of triabin. Higher concentrations up to a 67-fold molar excess did not lead to an increase of this inhibitory effect. This is in contrast to hirudin, which totally blocked the amidolytic activity of thrombin at low concentrations (Fig. 4). Other serine proteases, e.g. trypsin and factor Xa, were not inhibited in an amidolytic assay even when using a 45-fold molar excess of triabin (data not shown).

These results demonstrate that triabin is specific for thrombin. The difference in its inhibitory activity using either fibrinogen or a small synthetic peptide as substrate indicates that it may exert its inhibitory effect by binding to the anion-binding exosite rather than to the catalytic site of thrombin.

Limited trypsin proteolysis converts α-thrombin into β-thrombin through cleavage of the Arg73-Asn74 bond within the anion-binding exosite (23). Preincubation of thrombin with triabin prevented the cleavage by trypsin as analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). Since triabin did not inhibit trypsin, the effect was probably achieved by binding of triabin to the exosite of thrombin.

Thrombomodulin is known to exhibit its effect on thrombin by binding to the anion-binding exosite (6). The resulting change of substrate specificity enables thrombin to activate protein C. This activity can be tested by incubating thrombomodulin and thrombin with the inactive preform of protein C. This leads to activation of protein C which is detectable by its amidolytic activity. Triabin neither showed a thrombomodulin-like activity, i.e. it did not modulate thrombin, nor did it activate protein C. However, it was able to antagonize the effect of thrombomodulin in the protein C activation assay in a dose-dependent manner (Fig. 5). This observation further corroborates our hypothesis that triabin binds to this exosite of thrombin.

Twenty amino-terminal amino acids of triabin were sequenced and additional internal sequences were determined after cleavage with endoproteinase Lys-C and separation of the fragments by high performance liquid chromatography. Degenerate primers were made based on the protein sequence information and used to amplify a specific tag from T. pallidipennis salivary gland first-strand cDNA. The probe was employed to screen approximately 1 x 10^6 primary phage clones from a T. pallidipennis salivary gland cDNA library. DNA sequencing revealed that four different types of triabin cDNA had been isolated (Tr5, Tr12, Tr28, and Tr45). The clone with the longest insert is depicted in Fig. 6. It exhibited an open reading frame potentially coding for a 160-amino acid long protein. Hydrophobicity analysis showed that the 18 NH₂-terminal residues displayed the hallmark characteristic of a signal peptide (24). The following position was occupied by the alanine residue representing the NH₂ terminus of triabin isolated from T. pallidipennis saliva. Mature triabin is therefore a 142-amino acid protein.
probably glycosylated. Indeed a potential 15,948, slightly less than that of the saliva protein which is acid long protein with a predicted relative molecular mass of stretches previously determined by protein sequencing were retrieved in the amino acid sequence deduced from the cDNA. The insert sequence of the Tr5 clone is shown. The stretches previously determined by protein sequencing after Lys-C cleavage are highlighted with a dashed line. The nine amino acid positions where variations were found are underlined. The polyadenylation signal is shown. The identity was further checked by determination of the NH2-terminal sequence and the protein was found to start with the expected alanine residue, indicating that correct processing had taken place. The biological activity was evaluated in a platelet aggregation assay (Fig. 2). A dose-response study showed that recombinant triabin inhibited aggregation induced by 1 nM thrombin in a dose-dependent manner, the IC50 being 0.82 nM. A kinetic analysis of thrombin-catalyzed fibrinogen cleavage was performed by measuring fibrinopeptide A release, and the K1 was found to be 14.0 ± 3.7 μM. The inhibitory effect of recombinant triabin was assessed in this test, and the K1 measured. Since in this analysis, the amount of inhibitor bound by the enzyme was no longer negligible, the graphical method of Dixon (21) was used as it avoids the assumption that the total concentration of inhibitor is equal to the theoretically required free concentration and is therefore also applicable for tight-binding inhibitors. The K1 was found to be 3 μM (Fig. 7).

**DISCUSSION**

Blood-sucking insects employ various mechanisms to interfere with the blood coagulation of their host species. The saliva of T. pallidipennis inhibits collagen-induced platelet aggregation by pallidipin (8) and ADP-mediated aggregation by an...
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Similar experiments were performed with hirudin and its COOH-terminal fragment, which likewise inhibited the cleavage (29), and x-ray diffraction analysis showed that indeed these peptides bind to the anion-binding exosite (30). These results lend additional support to our hypothesis that triabin binds to thrombin in a similar manner.

Binding of thrombomodulin to thrombin causes a change in the specificity of the protease. Hofsteenge et al. (31) demonstrated that thrombomodulin inhibits the binding of fibrinogen to thrombin but hardly affects the binding of peptide substrates. Cleaving \( \alpha \)-thrombin at the Arg\(^{73}\)-Asn\(^{74}\) bond reduces its affinity to thrombomodulin (6). Additionally, an antibody binding to residues 62–73 of thrombin inhibits the activation of protein C by \( \alpha \)-thrombin in the presence of thrombomodulin (6). These results indicate that the region around Arg\(^{73}\), the anion-binding exosite, is necessary for the interaction with thrombomodulin. Therefore, it was of interest to investigate the effect of triabin on this interaction as well. Indeed, triabin inhibited in a dose-dependent manner the thrombomodulin-mediated activation of protein C by thrombin. This result further corroborates the hypothesis that triabin binds to the anion-binding exosite.

Four different cDNA clones with minor polymorphisms that probably do not affect the biological function of triabin were isolated. This diversity might reflect the existence either of a protein family or more likely of a genetic heterogeneity among the insects from which the salivary glands were collected. No sequence homology was found between triabin and the thrombin inhibitors hirudin and rhodniin. This is in keeping with the different modes of action of these proteins.

A vector for periplasmic expression was used for the production of triabin, in order to favor correct folding of the protein and facilitate the subsequent purification. Our results show that in both the fibrinogen clotting test and the thrombin-induced platelet aggregation assay, the biological activity of recombinant triabin purified from the periplasm of E. coli was similar to that of saliva triabin, suggesting that the protein has assumed its natural configuration.

The inhibition constant of 3 pM, which was measured in the thrombin-catalyzed fibrinogen cleavage assay, indicates a very tight binding of triabin to thrombin. For the hirudin-thrombin complex, dissociation constants of 63 pM (32), 3 pM (33), and 20 fm (34) have been cited in the literature. All these values were determined with an amidolytic substrate which could not be used for this study because triabin does not seem to block the active site. However, the value for triabin agrees well with the \( K_i \) found by Dodt et al. (33). In contrast, the COOH-terminal hirudin peptide Hir\(^{53}\)-64, which, like triabin, recognizes the anion-binding site of thrombin, has been shown to be less active in the thrombin-catalyzed hydrolysis of fibrinogen, the \( K_i \) being 144 nM (35).

In summary, we have purified a thrombin inhibitor with a potency comparable to hirudin but with a different mode of action. The availability of recombinant triabin will now allow us to further explore its mechanism of action and carry out structure-function studies using site-directed mutagenesis in order to identify the structural elements involved. X-ray diffraction analysis should provide detailed information on the structure of the triabin-thrombin complex. Furthermore, in vivo models will be tested to assess the effectiveness of triabin as an antithrombotic.

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2 C. Noeske-Jungblut, unpublished results.

Fig. 7. Effect of triabin on the steady-state velocity of thrombin. Assays were performed with 75 pM thrombin and 30 \( \mu \)M fibrinogen as described under "Experimental Procedures." Using the graphical method of Dixon (21), the \( K_i \) was determined.
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