Chromogenic substrates as fundamental tool to design new thrombin inhibitors

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Summary
The structure-activity relationship of dipetalogastin II, the strongest thrombin inhibitor isolated and cloned from the bug Dipetalogaster maximus, was examined by introducing gradual changes into the molecule by means of molecular biological methods. The effect upon its inhibition equilibrium constant was determined after each change by a chromogenic assay. This structural information was fundamental to design new dipetalogastin II-derived inhibitors. Our results suggested that the acidic sequence DEHDHDFEDT corresponding to amino acid residues 49 to 58 of dipetalogastin II reacts with the anion binding exosite (ABE) 1 of thrombin. Based on this finding, we constructed a chimeric molecule consisting of the active site blocking segment of dipetalogastin II (amino acid residues 1 to 48) and the ABE 1 blocking segment of hirudin. This construct showed better thrombin inhibitory activity than both separated segments only after the introduction of a glycine linker between both blocking segments. We thus obtained a thrombin inhibitor called dipetarudin with an inhibition equilibrium constant comparable to that of dipetalogastin II and a molecular mass below that of dipetalogastin.

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The importance of blocking thrombin activity in prophylaxis of thromboembolic disorders in high-risk patients has been extensively described. Coumarin (warfarin), heparin and its derivatives are the standard anticoagulants used for treatment and prevention of deep vein thrombosis and for the prophylaxis of cardiac disorders and stroke with thrombotic background. They have been in clinical use for over half a century, but both have serious drawbacks that limit their safe and efficacious use.

Animals depending on a diet of ingested fresh blood have mechanisms that interfere with the coagulation system of the blood victim. Their saliva contain anticoagulant compounds that inhibit the coagulation cascade at different steps, among which the thrombin inhibitors are the most prominent. Various thrombin inhibitors have been isolated from blood sucking parasites. Hirudin, isolated from the European leech Hirudo medicinalis is the most widely studied of them and has been characterised as a specific slow, tight-binding thrombin inhibitor with an inhibition equilibrium constant (Kᵢ) of 21 fmol/l and a molecular mass of about 7 kDa. Recombinant hirudin (r-hirudin) exhibits a 10-fold reduced affinity for thrombin (Kᵢ = 217 fmol/l) (6, 14).

Another potent thrombin inhibitor, Dipetalogastin II (DII), was isolated and cloned from the bug Dipetalogaster maximus (3, 7). It is a protein of 106 amino acid residues and a molecular mass of 12.9 kDa, which displays a two-domain structure. Biochemical characterization of recombinant DII (rDII) demonstrated that it acts as a slow, tight binding inhibitor of thrombin, with an inhibitory activity stronger than recombinant hirudin (Kᵢ = 49.3 fmol/l).

Recently, we have cloned a smaller dipetalogastin II-derived thrombin inhibitor, called dipetarudin, by replacing the bulky second head structure of dipetalogastin II by the anion binding exosite (ABE) 1 blocking segment of hirudin in order to decrease the high molecular mass of dipetalogastin II, but retaining its potent thrombin inhibitory activity. Biochemical characterization of dipetarudin revealed that it is also a slow, tight-binding inhibitor with a Kᵢ value of 446 fmol/l, which is comparable to that of rDII, but with a molecular mass of only 7.5 kDa (4).

Dissoziationskonstanten neuer Thrombininhibitoren: Bestimmung von Chromogene Substraten als Basis zur Entwicklung gleichbarer Dissoziationskonstanten

Schlüsselwörter
Direkt thrombin inhibitor, Ki-Wert, chromogenes Thrombinsubstrat

Zusammenfassung
Here, we describe how we studied the structure-activity relationship of dipetalogastin II. We introduced gradual changes by molecular biological methods and noted their effect upon thrombin inhibitory activity. The inhibitory activity of each dipetalogastin-derived variant was monitored by determination of its inhibition equilibrium constant for thrombin using a chromogenic assay. We also describe how this information allowed us to design the potent thrombin inhibitor dipetarudin.

Material and methods
Materials
Human α-thrombin was supplied by Kordia Laboratory Supplies, the Netherlands. The thrombin chromogenic substrate H-D-Phe-Pip-Arg-p-nitroaniline (S2238) was purchased from Amersham Biosciences, UK. Primers DIPCHI-R1 and DIPCHI-R2 as antisense primers (Tab. 1). All primers were designed according to the internal sequence of dipetalogastin II and only DIPCHI-R1 and DIPCHI-R2 also include the C-terminal sequence of hirudin.

The PCR products were subcloned into the expression vector pGEX-5X-1, using T4 DNA ligase and transformed in E. coli JM105. Gene expression was induced by adding isopropyl-b-D-thiogalactoside (IPTG). The resulting fusion proteins were purified using the affinity matrix Glutathione SepharoseTM 4B and cleaved with FXa. The released proteins were further separated and purified by RP-HPLC as described (4). Finally, the identity of each variant of dipetalogastin was checked by determination of its molecular mass using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) analysis in a Kompact Probe (Kratos Analytical, UK).

Inhibition equilibrium constant
The assay was performed in 0.05 mol/l Tris/HCl buffer (pH 8.0) containing 0.1 mol/l NaCl and 0.1% PEG 6000, inhibitor (at different concentrations) and S-2238 (final concentration: 77.5 pmol/l). The reaction was started by addition of 50 µl of α-thrombin (final concentration: 77.5 pmol/l). The progress curve of formation of p-nitroaniline was recorded spectrophotometrically at 405 nm over 10 min on a M400 spectrophotometer.

The inhibition equilibrium constant of each inhibitor (K_i) was determined by nonlinear regression analysis according to the theory of slow, tight binding or slow binding inhibition (8).

The progress curves obtained for several concentrations of each slow binding inhibitor were fitted by nonlinear regression to Equation 1.

\[ P = \frac{v_o t + (v_o - v_s) (1 - e^{-k_{obs} t})}{k_{obs} + d} \]  

where \( v_o, v_s \) 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 \( t \) and \( d \) is a displacement term to account for the fact that at \( t = 0 \) the absorbance may not be accurately known.

The values of \( v_o \) obtained from Eq. 1 were fitted to Eq. 2 to obtain the \( K_i \).

\[ v_o = \frac{V_{\max} \cdot [S]}{K_m + ([I] + [S])} + k_{off} \]  

The values of \( k_{obs} \) were fitted to Eq. 3 to obtain the association rate constant (\( k_{on} \) from the slope of this curve.

\[ k_{obs} = \frac{k_{on} (1 + [S]/K_m)}{[I] + k_{off}} \]  

Finally, from the relationship: \( K_i = k_{off}/k_{on} \), the \( k_{off} \) was calculated.

The mathematical analysis for slow tight binding inhibitors was done as described previously (4).

The Michaelis-Menten constant (\( K_m \)) of the chromogenic substrate (S-2238) was determined as follows: S-2238 was incubated at different concentrations (1.5, 3, 4.5, 7.5, 15 and 30 mol/l) in 0.05 mol/l Tris/HCl buffer (pH 8.0) containing 0.1 mol/l NaCl and 0.1% polyethylene glycol 6000. The reaction was started by adding thrombin. The initial velocity of thrombin catalyzed hydrolysis of S-2238 was recorded at 405 nm/l. All the hydrolytic reactions were performed at 25 °C. The initial reaction velocities (\( v_o \)) were plotted against the substrate concentrations ([S]) and then fitted to the Michaelis-Menten equation (Eq. 4) by nonlinear regression analysis.

\[ v_o = \frac{V_{\max} [S]}{K_m + [S]} \]  

### Cloning and purification of dipetalogastin II-derived inhibitors

The dipetalogastin cDNA cloned into the plasmid pV/6 (7) was used as template to amplify several fragments of the coding region of dipetalogastin II by polymerase chain reaction using different primers. EXP 1 was used as a sense primer and EXPR14, EXPR20, EXPR21, DIPCHI-R1 and DIPCHI-R2 as antisense primers (Tab. 1). All primers were designed according to the internal sequence of dipetalogastin II and only DIPCHI-R1 and DIPCHI-R2 also include the C-terminal sequence of hirudin.

The PCR products were subcloned into the expression vector pGEX-5X-1, using T4 DNA ligase and transformed in E. coli JM105. Gene expression was induced by adding isopropyl-b-D-thiogalactoside (IPTG). The resulting fusion proteins were purified using the affinity matrix Glutathione SepharoseTM 4B and cleaved with FXa. The released proteins were further separated and purified by RP-HPLC as described (4). Finally, the identity of each variant of dipetalogastin was checked by determination of its molecular mass using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) analysis in a Kompact Probe (Kratos Analytical, UK).

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### Methods

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### Theory of slow, tight binding or slow binding inhibition

The progress curves obtained for several concentrations of each slow binding inhibitor were fitted by nonlinear regression to Equation 1.

\[ P = \frac{v_o t + (v_o - v_s) (1 - e^{-k_{obs} t})}{k_{obs} + d} \]

where \( v_o, v_s \) 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 \( t \) and \( d \) is a displacement term to account for the fact that at \( t = 0 \) the absorbance may not be accurately known.

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\[ v_o = \frac{V_{\max} [S]}{K_m + [S]} \]
Results, discussion

Different recombinant N-terminal fragments of dipetalogastin II containing the first 68, 58 or 48 amino acids were cloned and designated as rDII_{1–68}, rDII_{1–58} and rDII_{1–48}, respectively. The amplification of all these variants of dipetalogastin II was performed from the plasmid V/6 that contains the cDNA of dipetalogastin I, II and III (7) using specific primers in PCR. DNA fragments were subcloned into the expression vector pGEX-5X-1, which allowed intracellular expression of each dipetalogastin variant as a fusion with GST. The fusion proteins were cleaved with FXa and recombinant dipetalogastin II variants were purified by HPLC (data not shown). The molecular masses of these variants determined by MALDI-TOF mass spectrometry were in agreement with those calculated by amino acid composition (data not shown).

The interaction of rDII-derived inhibitors with α-thrombin displayed slow-binding kinetics, evidence of this was the downward concavity displayed for the progress curve when α-thrombin was added to the reaction medium containing an rDII-derived inhibitor and the substrate S-2238. Moreover, rDII_{1–68}, rDII_{1–58} and rDII_{1–48} inhibited thrombin at a much higher concentration than the enzyme concentration indicating that they act as slow binding inhibitors of thrombin (9, 10, 17).

For each inhibitor a set of progress curves was obtained at several inhibitor concentrations. The data were fitted by nonlinear regression to Eq. 1 (see methods). The Figure 1a shows the results obtained with rDII_{1–58}. For each inhibitor concentration, it was observed that after a rapid initial phase, the initial velocity decreased to a slower steady-state. However, the initial velocity of the reactions was independent of the inhibitor concentrations.

The values of v_{o} obtained by Eq. 1 were then incorporated into Eq. 2 to obtain the K_{i} value (Fig. 1b). Furthermore, the parameter k_{obs} increased linearly with the inhibitor concentration (Fig. 1c). These values of k_{obs} were fitted to Eq. 3 to obtain the association rate constant (k_{on}) from the slope of this curve. The K_{i} value of the chromogenic substrate (S-2238) was determined prior to each K_{i} determination and varied between 1.9 and 2.4 µmol/l. The results of the kinetic analysis of each rDII-derived inhibitor are shown in Table 2.

The smallest N-terminal fragment of dipetalogastin II (rDII_{1–48}) has the poorest affinity for thrombin, as given by the increased value of the dissociation constant of the corresponding thrombin complexes (K_{i} of 641 pmol/l). A prolongation of 10 amino acids yields a molecule (rDII_{1–58}) that inhibits thrombin 32-fold more effectively than rDII_{1–48} (K_{i} of 20.24 pmol/l). Another increment of 10 amino acids in the sequence to lead rDII_{1–68} did not produce a further improvement in the affinity for α-thrombin (K_{i} value of 21.7 pmol/l).

Comparison of these kinetic parameters suggested that the acidic sequence DEHDHDFEDT (residues 49 to 58 of dipetalogastin II) seems to be those amino acids that react with ABE 1 of thrombin, which could explain the higher thrombin affinity of rDII_{1–58} in comparison with rDII_{1–48}. However, other interactions between the se-
cond head structure of dipetalogastin II and thrombin might be necessary to achieve the highest thrombin affinity observed in the full-length rDII.

It is very important to emphasize that all thrombin inhibitors found in the nature are bifunctional inhibitors. It means that they have a portion that binds the active site and simultaneously, another one that binds predominantly to the ABE 1 of thrombin (2, 13, 15, 16) or in the case of heamadin to the ABE 2 (12). Triabin is the only mono-functional inhibitor found in the nature. It binds only to the ABE 1 of thrombin (1).

Imitating the structure of natural thrombin inhibitors, bivalent synthetic thrombin inhibitors have been developed by adding the hirudin fragment that binds ABE 1 of thrombin to an active site blocking segment. Only this association with hirudin allowed an improvement of the specificity and the thrombin affinity of the synthetic inhibitors, probably due to the simultaneous binding of these inhibitors to the active site and the ABE 1 of thrombin. An example of bivalent synthetic thrombin inhibitors is hirulog-1, also called bivalirudin (5). It is a 20 amino acid peptide (dPhe-PRPGGGGDGFEEI-PEEYL) with an inhibition constant of 1.9 nmol/l. Hirulog-1 is the only compound of this class which has been in clinical development for the treatment of a variety of clotting disorders, however, due to that it has a very short half-life (26 minutes) and to that inhibits clot-bound thrombin by a reaction easily reversible, it is inefficient in the prevention of reocclusion after thrombolytic treatment.

Dipetalogastin II is also a bifunctional inhibitor, but in contrast to hirudin, it has a very bulky structure to interact with the ABE 1, corresponding to the amino acid residues 49 to 106. For this reason, we decided to replace these residues of dipetalogastin II by the ABE 1 blocking segment of hirudin (DFEEIPEEYLQ) leading to a smaller chimeric thrombin inhibitor with a K_i value of 12.85 pmol/l, which is 50-fold lower than that of rDII_1–48 and only slightly lower than those of rDII_1–58 and rDII_1–68.

Moreover, knowing that the distance between the active site and ABE 1 blocking segments is very important to ensure their optimal interaction with thrombin, another chimeric inhibitor containing a flexible linker of S-glycine residues between both binding segments was also cloned. The insertion of the linker resulted in a 29-fold decrease of the dissociation constant (K_i value of 0.45 pmol/l) compared to that of the chimeric inhibitor without linker. The low K_i value was chiefly due to an increase in the k_off value. This finding indicates that the glycine linker improved the association between the inhibitor and thrombin. This potent chimeric thrombin inhibitor was denominated dipetarudin (4).

The inhibition data obtained for dipetarudin corroborate that the interactions at the active site and at the ABE 1 are additive. It binds to thrombin with an affinity higher than that of rDII_1–48 (K_i of 641 pmol/l) and hirugen, the synthetic N-acetylated carboxy terminal dodecapeptide of hirudin (NGDFEEIPEEYL) that acts as a pure competitive inhibitor of thrombin-catalyzed release of fibrinopeptide A from human fibrinogen with a dissociation constant of 540 nmol/l (11).

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor class</th>
<th>k_i (mol/l) × 10^{-12}</th>
<th>k_m (mol·l⁻¹·s⁻¹) × 10^4</th>
<th>k_cat (s⁻¹) × 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>rDII_1–48</td>
<td>SB</td>
<td>21.70 ± 13.42</td>
<td>6.47 ± 0.37</td>
<td>1.38 ± 0.81</td>
</tr>
<tr>
<td>rDII_1–58</td>
<td>SB</td>
<td>20.24 ± 14.12</td>
<td>4.14 ± 0.69</td>
<td>0.84 ± 0.62</td>
</tr>
<tr>
<td>rDII_1–68</td>
<td>SB</td>
<td>641 ± 92.72</td>
<td>0.66 ± 0.05</td>
<td>4.16 ± 0.75</td>
</tr>
<tr>
<td>Chimera without linker</td>
<td>SB</td>
<td>12.85 ± 4.13</td>
<td>14.27 ± 3.04</td>
<td>1.75 ± 0.26</td>
</tr>
<tr>
<td>Chimera with linker</td>
<td>STB</td>
<td>0.45 ± 0.08</td>
<td>842.3 ± 178.16</td>
<td>3.66 ± 0.46</td>
</tr>
</tbody>
</table>

### Conclusion

The structure-activity relationship of the potent thrombin inhibitor dipetalogastin was examined by introducing gradual changes into the molecule and examining the effect upon the inhibition equilibrium constant after each change using a chromogenic assay. This colorimetric method was very sensitive for detecting changes in the structure of dipetalogastin II-derived inhibitors and allowed us to choose the best derivative for future investigations.

### References

12. Richardson J, Kröger B, Hoeftken W et al. Crystal structure of the human ocomplex: an exo-


15. van de Locht A, Lamba D, Bauer M et al. Two heads are better than one: crystal structure of the insect derived double domain Kazal inhibitor rhodnin in complex with thrombin. EMBO J 1995; 14: 5149–57.


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