Lebetin Peptides: Potent Platelet Aggregation Inhibitors

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Key Words
Snake venom · Lebetin peptides · Antiplatelet aggregation · Thrombocytopenia

Abstract
Lebetins from Macrovipera lebetina snake venom constitute a new class of inhibitors of platelet aggregation. There are two groups of peptides: lebetin 1 (L1; 11- to 13-mer) and lebetin 2 (L2; 37- to 38-mer). The short lebetins are identical to the N-terminal segments of the longer ones. They inhibit platelet aggregation induced by various agonists (e.g. thrombin, PAF-acether or collagen). The shortest lebetin (11-mer) shows potent inhibition of rabbit (IC₅₀ = 7 nM) and human (IC₅₀ = 5 nM) platelets. They prevent collagen-induced thrombocytopenia in rats. N- and C-terminal-truncated synthetic L1γ (sL1γ; 11-mer) is less active in inhibiting platelet aggregation than the native peptide. Results from Ala scan studies of the sL1γ peptide indicated that replacement of the residues (P3, G7, P8, P9 or N10) resulted in a remarkable drop in the activity, whereas replacement of residues K2, P4 or K6 by Ala resulted in enhancement of the antiplatelet activity by at least 10-fold. To examine the activity of multimeric L1γ, several multimeric peptides were synthesized using the multiple-antigen peptide system assembled on a branched lysine core and their antiplatelet activity was evaluated in vitro. The largest multimeric peptides showed a 1,000-fold increase in antiplatelet activity.

Introduction
Platelet aggregation plays a fundamental role in hemostasis as it arrests bleeding through the initial hemostatic plug that forms at the site of vascular injury [1]. Platelet aggregation involves a complex network of cell surface adhesion proteins, one of which is GPIIb/
Table 1. Amino acid sequences and molecular masses of lebetins

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Measured mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.305.74±0.38</td>
<td></td>
</tr>
<tr>
<td>1.248.69±0.31</td>
<td></td>
</tr>
<tr>
<td>1.191.45±0.43</td>
<td></td>
</tr>
<tr>
<td>3.943.74±0.46</td>
<td></td>
</tr>
<tr>
<td>3.886.92±0.38</td>
<td></td>
</tr>
</tbody>
</table>

IIIa. GPIIb/IIIa binds fibrinogen; their interaction is inhibited by proteins isolated from snake venom containing an RGD sequence [2]. Disintegrins, short RGD containing peptides such as echistatin, inhibit several integrins (e.g. αIIβ3, αvβ3, α5β1), whereas eristostatin is selective for αIIβ3 [3, 4]. The heterodimeric disintegrins are more specific for leukocyte integrins. Two of them, EC3 and EMF10, inhibit αvβ3/αvβ7 and α5β1, respectively [5, 6].

We isolated a new family of platelet aggregation inhibitors, lebetin 1 (L1) and lebetin 2 (L2), from *Macrovipera lebetina* venom. Despite the absence of RGD-related sequences or other sequences with known antiaggregating activity, lebetins displayed strong in vitro antiplatelet activity and were able to prevent collagen-induced thrombocytopenia in rats.

**Purification and Characterization of L1 and L2**

L1 and L2 were purified to homogeneity from *M. lebetina* venom in two steps of gel filtration followed by reverse-phase HPLC on a C8 column [7]. N-terminal sequencing of native L2 revealed two homologous sequences representing 80% (α) and 20% (β) of the total yield. The N-terminal Gly residue was deleted in the latter (table 1). Their molecular weights determined by ES-MS were 3,943.74 and 3,886.96, respectively. A minor L1γ was also found in *M. lebetina* venom (table 1). Complete sequences of L2α and L2β were deduced by sequencing the alkylated peptides and the C-terminal peptide obtained by digestion with Arg-C protease. The complete L2α and L2β isomers are shown in table 1. L1α, L1β and L1γ contained 13, 12 and 11 amino acid residues, respectively, whereas L2α and L2β contained 38 and 37 amino acid residues, respectively. The L1 isoforms are identical to the N-terminal sequences of the L2 isoforms. The lebetin peptides lack the RGD motif and do not share any significant structural similarity with known disintegrins.

**In vitro Platelet Aggregation Inhibition by Lebetins**

Rabbit platelet aggregation induced by thrombin, PAF-acether or collagen was inhibited by L1 with IC50 values of 125, 48 and 27 nM, respectively, and L2 with IC50 values of 8, 48 and 5 nM, respectively. L1 and L2 also inhibited thrombin-induced aggregation of washed human platelets with IC50 values of 590 and 100 nM, respectively.
In vivo Activity of Lebetins

Intravenous administration of L1 and L2 inhibited collagen-induced thrombocytopenia with ED_{50} values of 2 and 4.2 nmol/kg, respectively. L1 (20 nmol/kg) and L2 (13 nmol/kg) reduced the thrombocytopenia by 80–90%.

Structure-Activity Relationships

To determine the relative activity of each peptide, L1α, L1β, L1γ, L2α, L2β were synthesized and their antiplatelet activities determined. The synthetic short and long lebetin peptides inhibited thrombin-induced rabbit and human platelet aggregation in the nanomolar range (table 2). Various L1γ-derived synthetic peptides, truncated at either their N- or C-terminus by one or more amino acid residues, were synthesized. The antiplatelet activity of truncated peptides was drastically decreased showing that the integrity of synthetic L1γ (sL1γ) is essential for its potent effect. The results from Ala scan of sL1α demonstrated that substitution of P3, G7, P8, P9 or N10 drastically decreased the antiplatelet activity of the peptides. However, a significant antiplatelet activity is retained when N1, K5 are individually replaced by Ala (IC_{50} = 50 nM instead of 7 nM for sL1α). The antiplatelet activity increased by at least 10-fold when K2, P4 or K6 were replaced by Ala.

Another way to dramatically increase the activity is to prepare multimeric peptides. Several sizes of multimeric sL1γ peptides were chemically synthesized using the multiple-antigen peptide system assembled on a branched lysine core and fully characterized. The multimeric peptides were found to be 1,000-fold more active than the sL1γ peptide in inhibiting rabbit thrombin-induced platelet aggregation, with IC_{50} values ranging from 2 to 25 pM. The larger the peptide, the more inhibitory effect there was on platelet aggregation.

Conclusion and Perspectives

Native and synthetic lebetins possess potent antiplatelet activity and prevent thrombocytopenia induced by collagen in rats. Structure-activity relationship studies of sL1γ showed the importance of its integrity and the possibilities of enhancing the potency by alanine replacement or by synthesis of multimeric forms. However, the antiplatelet mechanism of action of lebetin remains to be elucidated and its in vivo antithrombotic effects evaluated.

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Table 2. Effect of synthetic lebetin on rabbit and human platelet aggregation induced by thrombin (0.04 IU/ml)

<table>
<thead>
<tr>
<th>Synthetic peptides</th>
<th>Rabbit platelets IC_{50}, nM</th>
<th>Human platelets IC_{50}, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>sL1α</td>
<td>23</td>
<td>140</td>
</tr>
<tr>
<td>sL1β</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>sL1γ</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>sL2α</td>
<td>0.2</td>
<td>2.5</td>
</tr>
<tr>
<td>sL2β</td>
<td>0.9</td>
<td>2.8</td>
</tr>
</tbody>
</table>

IC_{50} = Concentration of inhibitor required to inhibit 50% aggregation.
References