Novel anti-platelet aggregation polypeptides from *Vipera lebetina* venom: isolation and characterization

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Abstract Lebetins 1 and Lebetins 2, two polypeptide groups that inhibit platelet aggregation, were isolated from *Vipera lebetina* venom by gel filtration and reverse phase chromatography. Amino acid sequencing indicated that the first group contains two major polypeptides of 13 and 12 residues; their molecular weight was determined by electrospray mass spectrometry. The second was composed of two peptides of 38 and 37 residues, each with one disulfide bond. Sequence analysis revealed neither RGD sequence nor homology with other proteins including known snake or tick polypeptides. Lebetins 1 were Pro and Lys rich peptides and their sequences were identical to the N-terminus of Lebetins 2. Lebetins inhibited platelet aggregation induced by thrombin, collagen and PAF-acether. The 50% concentration that inhibited human and rabbit platelet aggregation induced by thrombin was 590 nM and 125 nM for Lebetins 1 and 100 nM and 8 nM for Lebetins 2, respectively. Lebetins 1 and Lebetins 2 also inhibited fibrinogen-induced aggregation of α-chymotrypsin-treated platelets as well as in vivo collagen-induced thrombocytopenia in rats with half effective doses of 2 nmol/kg and 4.2 nmol/kg, respectively. Lebetins were not toxic after intravenous injection into mice and rats. These polypeptides form novel platelet inhibitors that could be used to delineate further the mechanisms of platelet aggregation and serve as a model for developing antithrombotic agents.

Key words: Aggregation; Antithrombotic; Platelet; Snake; Thrombocytopenia; Venom

1. Introduction

Several components isolated from snake venoms – e.g. platelet aggregation inhibitors, phospholipases and ADPases, thrombin-like enzymes, fibrinolytic enzymes – affect hemostasis by interfering with the coagulation and platelet aggregation processes [1–5]. Platelet aggregation involves a complex network of cell surface adhesion proteins, one of which is GPIIb/IIIa. GPIIb/IIIa binds fibrinogen and this binding is inhibited by proteins isolated from snake venom and containing an RGD sequence [1]: the 'disintegrins'. Fibrinogen-GPIIb/IIIa interaction is the final step of a complex cascade of biochemical reactions [6] and cell morphological changes, including activation of platelets which become competent to bind fibrinogen, changes in shape, secretion of the granular content and aggregation. These events are induced by platelet aggregation agonists [7,8] and each of these may be a target for anti-aggregation agents.

We isolated from *Vipera lebetina* venom two groups of peptides, Lebetins 1 and Lebetins 2. Despite the absence of either RGD-related sequences or other sequences with known anti-aggregating activity, both groups displayed a strong in vitro antiplatelet activity and were able to prevent in vivo collagen-induced thrombocytopenia in rats.

2. Materials and methods

2.1. Materials

Venoms were obtained from mature *Vipera lebetina* (serpentarium of the Institut Pasteur, Tunis) and stored at −20°C until use. Rabbits (HY/CR), Swiss mice and rats (Wistar) were from the Institut Pasteur (Tunis). Human thrombin (R3235) was from Hoffmann La Roche Diagnostica (Basel, Switzerland), collagen and PAF-acether from Chronolog (Havertown, PA, USA), EGTA and α-chymotrypsin from Sigma (St. Louis, MO, USA), fibrinogen from KabiVitrum (Stockholm, Sweden), pentobarbital from Sanofi (Paris, France), sequencing grade Arg C protease from Boehringer GmbH (Mannheim, Germany). HPLC columns were from Beckman (Fullerton, CA, USA), Sephadex G-75 from Pharmacia (Uppsala, Sweden), the aggregometer from Coultronics (Chronolog 560 CA; Margency, France), the 476 A sequencer from Applied Biosystems Inc. (Foster City, CA, USA), the amino acid analyzer from Beckman (Mo. 6300; Palo Alto, CA, USA).

2.2. Purification of Lebetins

A pool of venom (2 ml; 385 absorbance units at 280 nm) was gel-filtered on a Sephadex G-75 column (2.6×100 cm; flow rate: 16 ml/h; fraction: 3 ml) in 200 mM ammonium acetate, pH 6.8. The fractions with anti-aggregation activity (determined as described below on thrombin-induced rabbit platelet aggregation) were further purified by analytical reverse phase HPLC on a C8 column (250×4.6 mm, 5 μm) equilibrated in 5% acetonitrile/0.1% trifluoroacetic acid (TFA). Elution was performed using a linear gradient (5–70%) of acetonitrile/0.1% TFA in 40 min (1 ml/min) and monitored by measuring the absorbance (214 nm) with a Beckman 166 detector. The homogeneity of peptide fractions displaying anti-aggregation activity was checked on a C18 column (250×4.6 mm, 5 μm) in the conditions described above. Venom proteins were quantified by the enhanced alkaline copper ('Lowry') protein assay [9] and by absorbance at 280 nm. Peptides were quantified from amino acid composition.

2.3. Protein analysis

S-Pyridylethylated Lebetins 2 (10 nmol) were prepared by reduction of the disulfide bond with dithiothreitol (a 60-fold molar excess over the *S-S* bond in 6 M guanidine, 250 mM Tris-HCl pH 8.5, 4 mM EDTA, under nitrogen, at 40°C for 20 h in the dark) and *S*-alkylation by adding 4 vinylpyridine for 20 min at 20°C. Alkylated and desalted Lebetins 2 (2 nmol) were digested with 2% (w/w) Arg C protease for 20 h in 100 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$, at 37°C. The reaction was stopped by freezing. The resulting peptides were purified on a 140 HPLC system equipped with a C18 PTH column (2.1×220 mm, 5 μm, Applied Biosystems). After an initial isocratic step for 3 min, a gradient of 1.5–62% acetonitrile/0.1% TFA was applied for 60 min (flow rate: 200 μl/min). Purified Lebetins 1 and Lebetins 2 were subjected to Edman automated sequencing according to the manufacturer's instructions. Amino acid analyses of native or alkylated proteins were performed after hydrolysis at 110°C for 20 h or at 150°C

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for 1 h in evacuated tubes. Electrospray mass spectrometry (ESMS) of native Lebetins 1, and native or alkylated Lebetins 2 (5 μM in water/methanol (50:50, v/v) containing 1% acetic acid) was performed on a VG-BIO-Q (Bio-tech) in the positive mode [10].

2.4. Platelet preparation and in vitro aggregation assay

Rabbit platelets were prepared from a 0.2 M EDTA-treated blood sample [11]. Human platelets were prepared as in [12]: blood was collected in vials containing a sodium citrate/dextrose (1:5 v/v) mixture after venipuncture of healthy volunteers free of drugs for at least 7 days. Platelets were resuspended in Tyrode's buffer pH 7.4 at a final concentration of 3×10^8 cells/ml prior to the assay that was performed at 37°C with stirring in an aggregometer. For anti-aggregation activity assays, washed platelets (1.2×10^8 cells/400 μl) were incubated at 37°C for 2 min with Lebetins, and then stimulated with agonists (thrombin: 0.04 IU/ml; PAF acether: 10^{-7} M; collagen: 5 μg/ml). The aggregation was monitored by recording the change in light transmission. The concentration of peptide (an average molecular weight was calculated for each peptide group and used thereafter in the study) giving 50% inhibition of platelet aggregation (IC_{50}) was determined from the linear portion of the dose-response curve.

Rabbit platelets prepared as described above were treated with α-chymotrypsin (8 IU/ml) for 15 min at 22°C [13] and then resuspended (3×10^8 cells/ml). Human fibrinogen (0.125–1.5 mg/ml) was added with or without Lebetins to the washed α-chymotrypsin-treated platelets and the mixture was incubated for 7 min at 37°C with stirring.

2.5. In vivo inhibition of collagen-induced thrombocytopenia in rats

This procedure was performed as in [14] with modifications. Rats (200 g) were anesthetized with sodium pentobarbital (1 mg/kg). Catheters were inserted into the jugular vein and the carotid. Lebetins (0–50 μg/kg of body weight dissolved in 0.9% NaCl) were injected into the left jugular. One minute later, 1 ml of blood was collected within 30 s from the right carotid into a vial containing an EDTA (4.5 M)/Tris (0.1 M)/indomethacin (0.15 M) pH 7.4 solution. Platelet rich plasma (PRP) was prepared by centrifugation ($200\times g$, 10 min, 22°C) and platelets were counted. Two minutes after the injection of Lebetins, 1 mg of collagen/kg (a dose sufficient to lower the normal platelet count by 60%) was injected into the left jugular vein. One minute later, 1 ml of blood was collected from the right carotid and platelets were counted. Inhibition of the collagen-induced thrombocytopenia was calculated by comparison of platelet counts observed in animals receiving Lebetins versus those receiving saline solution. The effective doses giving 50% inhibition of thrombocytopenia (ED_{50}) were calculated by linear regression of the linear portion of the dose-response curves.

2.6. Toxicity

Groups of six Swiss mice (20 ± 2 g) were injected intravenously with 50 μg of Lebetins 1 or Lebetins 2 dissolved in saline solution pH 7.4. Toxicity was scored after 24 h.

3. Results

3.1. Purification of Lebetins

G-75 gel filtration of the crude venom yielded five protein fractions. Fig. 1A shows a typical ($n=12$) profile of the fractionated venom; the yield of the column was about 98%. Fractions II and particularly IV strongly inhibited thrombin-induced rabbit platelet aggregation. Characterization of fraction II will be reported elsewhere. Here, we described the purification of fraction IV which contained low molecular weight peptides and represented 1% of the absorbance of the proteins in the crude venom. Fraction IV was investigated by reverse phase HPLC on C8 column; five peaks were obtained (Fig. 1B is a typical run; $n=5$). Two peaks with retention times of 8.25 and 14.02 min were potent antiplatelet agents: they were named Lebetins 1 and Lebetins 2, respectively. They represented about 13% and 10% of the absorbance at 214 nm of fraction IV. Lebetins 1 and Lebetins 2 appeared to be homogeneous as judged by further analysis by C18 reverse phase HPLC (Fig. 1C,D).

3.2. Protein analysis of Lebetins

N-terminal sequencing of native Lebetins 2 revealed two homologous sequences representing 80% (α) and 20% (β) of the total yield. The latter was deleted in the N-terminal part by a Gly residue. The two sequences showed 4 gaps in each (Fig. 2Aa). Their molecular weights were determined by ESMS (Table 1) and were 3943.74 ± 0.46 and 3886.92 ± 0.38, the difference (56.82 ± 0.84) being consistent with the absence of a Gly residue in β. Assuming similar ionization states for these two homologous compounds [15], their relative amounts estimated by ESMS agreed with proportions deduced from sequencing. Reverse phase chromatography failed to separate Lebetins 2α and β reduced and alkylated isoforms as they differ only by a Gly residue. Complete primary structures were deduced by sequencing the alkylated Lebetins 2α and β and a C-terminal peptide (Fig. 2Ab,c) obtained by digestion with Arg C protease and elution from a microbore C18 column (retention time = 28.24 min). The complete sequences of Lebetins 2α and β isoforms are shown in Fig. 2Ad,e. Each isoform was reticulated by one disulfide bond. These results are in agreement with those obtained by ESMS, for the native as well as for the alkylated forms (Table 1). Native Lebetins 1 were also sequenced. Two major peptides, α and β (Fig. 2B), and a minor one, γ, were detected; α and β differed by a Gly residue at their N-terminus and represented 30 and 60% of Lebetin 1 isoforms, respectively. Their molecular weights determined by ESMS were 1305.74 ± 0.38 and 1248.69 ± 0.31, respectively, and the difference (57.05 ± 0.69) is consistent with an additional N-terminal Gly residue in Lebetin 1α (Fig. 2Bf). The calculated proportions deduced from ESMS measurements were 19% and 68%, respectively. There is a good agreement between the calculated and the measured masses and the relative amounts of Lebetins 1α and β. A third minor isoform, Lebetin 1γ (less than 10%), was detected. Le-

Table 1
Amino acid sequences and molecular masses of Lebetins

Lebetin isoform	Proposed sequence	Form	Calculated mass	Measured mass
Lebetin 2α	GDNKPPKKGPPNGCFGHKI-DRIGSHSGLGCNKVDDNKG	Native	3943.39	3943.74 ± 0.46
		Alkylated	4155.68	4156.61 ± 0.77
Lebetin 2β	DNKPPKKGPPNGCFGHKI-DRIGSHSGLGCNKVDDNKG	Native	3886.33	3886.92 ± 0.38
		Alkylated	4098.63	4098.68 ± 0.25
Lebetin 1α	GDNKPPKKGPPNG	Native	1305.46	1305.74 ± 0.38
Lebetin 1β	DNKPPKKGPPNG	Native	1248.41	1248.69 ± 0.31

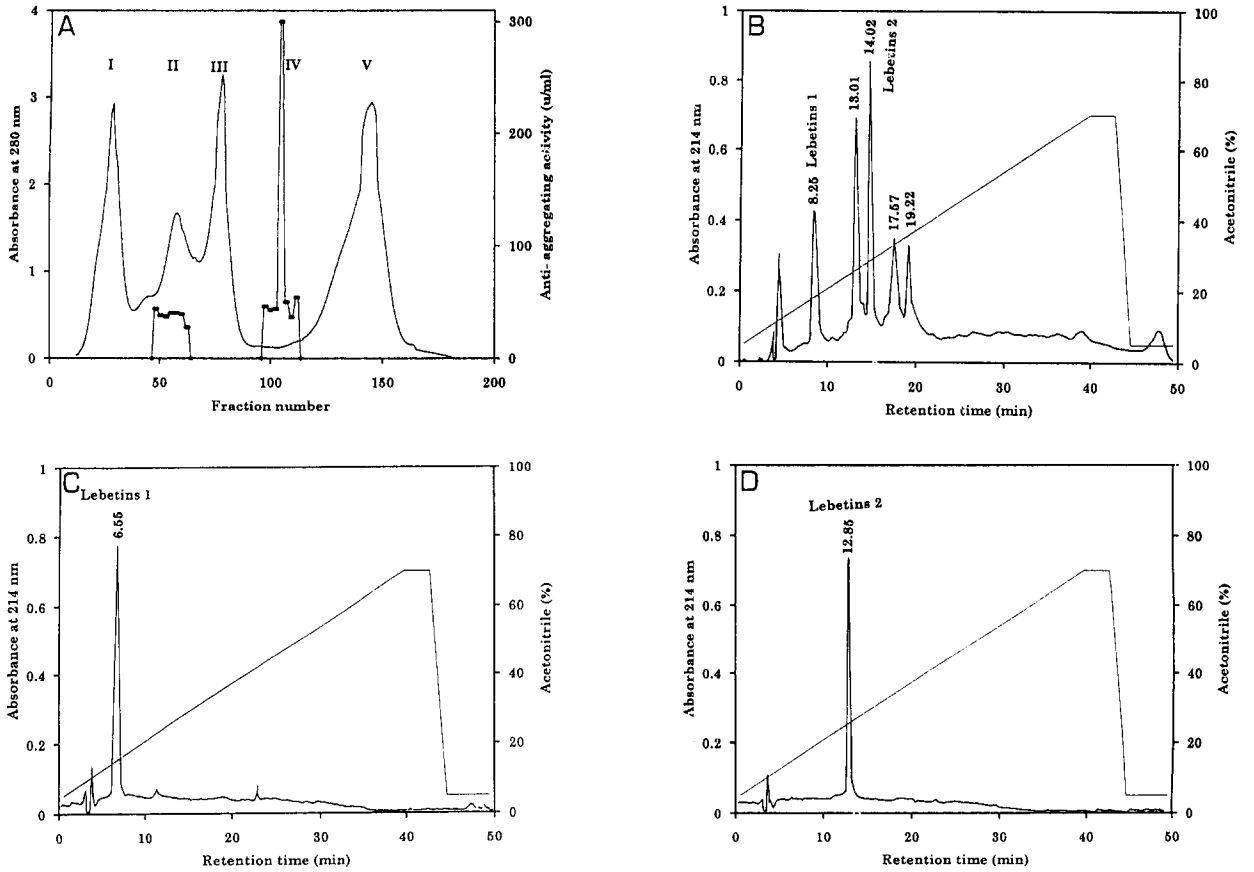


Fig. 1. Purification of Lebetins. A: G-75 gel filtration of the venom (line). The fractions were assayed for inhibition of rabbit platelet aggregation induced by thrombin: anti-aggregating units (U/ml) (●) were defined as 'absorbance at 280 nm per ml/IC_{50}'. B: Fraction IV was applied to a RP-HPLC C8 column. The fractions named Lebetins 1 (1C) and Lebetins 2 (1D) were further analyzed by a C18 column.

betins 1α and β were constituted by 13 and 12 amino acids respectively, and Lebetins 2α and β by 38 and 37 amino acid residues respectively. The amino acid composition analysis of Lebetins 1 and Lebetins 2 (data not shown) was in agreement with their respective deduced sequences. Lebetins 1 are identical to the N-terminal sequence of Lebetin 2 isoforms. Lebetins displayed no homology with known proteins, as determined by homology search in SwissProt, NBRF, PDB and GenPro sequence data base.

3.3. *In vitro platelet aggregation inhibition by Lebetins*

The crude venom of *Vipera lebetina* inhibited rabbit platelet aggregation induced by thrombin (0.04 IU/ml), PAF-acether (10^{-7} M) or collagen (5 μg/ml) with comparable IC_{50}s (data not shown; 70, 90 and 55 μg/ml, respectively, in a representative experiment ($n = 5$)). Rabbit platelet aggregation induced by thrombin, PAF-acether or collagen was also inhibited by Lebetins 1 with IC_{50}s of 125, 48 and 27 nM respectively (Fig. 3A) and Lebetins 2 with IC_{50}s of 8, 48 and 5 nM, respectively (Fig. 3B). Lebetins 1 and Lebetins 2 also inhibited thrombin-induced aggregation of human platelets with IC_{50}s of 590 nM and 100 nM, respectively (Fig. 3C).

Aggregation of α-chymotrypsin-treated platelets was also achieved with fibrinogen; 1.5 mg/ml of fibrinogen induced 50% of the maximum platelet aggregation induced by thrombin and Lebetins 1 (115 nM) or Lebetins 2 (43 nM) lowered this aggregation by 50% (data not shown from a representative experiment; $n = 5$).

3.4. *Inhibition by Lebetins of collagen-induced thrombocytopenia in rats*

Intravenous administration of Lebetins 1 and Lebetins 2 inhibited collagen-induced thrombocytopenia with ED_{50}s of 2.5 μg/kg (2 nmol/kg) and 17 μg/kg (4.2 nmol/kg), respectively, in a representative experiment ($n = 5$). Doses of 20 nmol/kg of Lebetins 1 and 13 nmol/kg of Lebetins 2 lowered the thrombocytopenia by 80–90% (Fig. 4).

3.5. *Toxicity*

No toxicity was observed upon the intravenous injection of Lebetins 1 and Lebetins 2 to mice.

4. Discussion

Vipera lebetina venom prevents the platelet aggregation induced by various agonists, with IC_{50}s between 55 and 90 μg/ml. Based on this observation, we isolated two polypeptide groups that we named Lebetins 1 and Lebetins 2. The Lebetins preparations obtained by two successive reverse phase HPLC steps appeared to be homogeneous. However, sequence and ESMS analysis revealed that:

1. Lebetins 2 contained two isoforms, α and β, representing respectively 80 and 20% of this group. They are 38 and 37 amino acid residues long, respectively. They contain one disulfide bond and differ by a single Gly residue present at the N-terminus of Lebetin 2α. This explains why they were not separated

A: LEBETINS 2

a GDNKPPKKGPPNG?F?HKIDRIGSH?GLG?NKVDDNK

DNKPPKKGPPNG?F?HKIDRIGSH?GLG?NKVDDNK

b GDNKPPKKGPPNGCFGHKIDRIGSH

DNKPPKKGPPNGCFGHKIDRIGSHS

c IGSHSGLGCNKVDDNKG

d GDNKPPKKGPPNGCFGHKIDRIGSHSGLGCNKVDDNKG (α)

e DNKPPKKGPPNGCFGHKIDRIGSHSGLGCNKVDDNKG (β)

B: LEBETINS 1

f GDNKPPKKGPPNG (α)

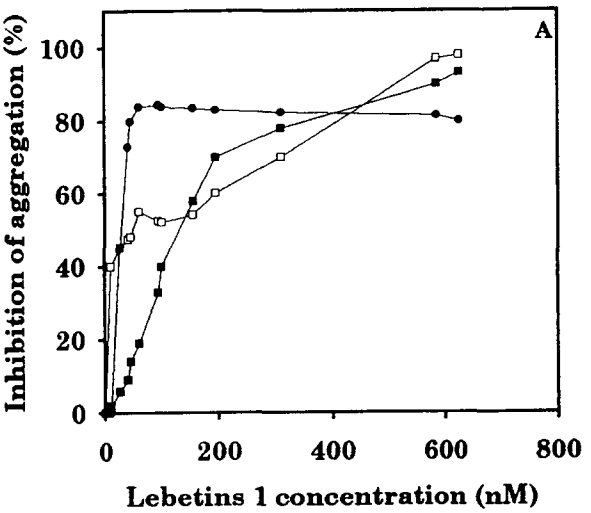
g DNKPPKKGPPNG (β)

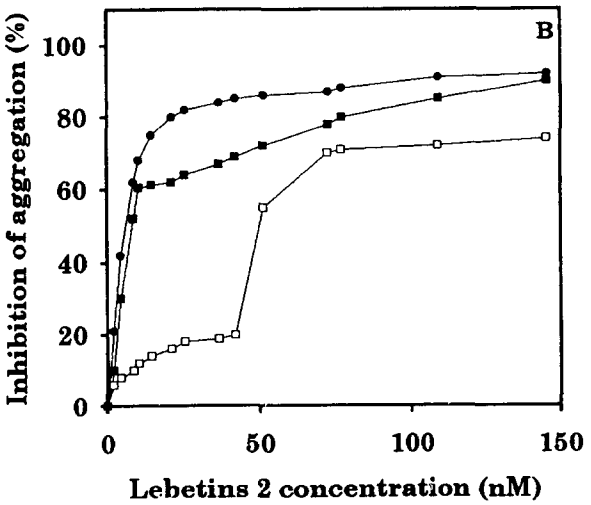
Fig. 2. Amino acid sequences of Lebetins. A: Sequences of Lebetins 2. a: native isoforms α and β; b: reduced and alkylated isoforms α and β; c: peptide (retention time: 28.24 min) obtained after Arg C proteolysis; d: deduced sequence of isoform α; e: deduced sequence of isoform β. B: Sequences of Lebetins 1. f: deduced sequence of isoform α; g: deduced sequence of isoform β.

2. the Lebetins 1 major isoforms α and β represented about 30% and 60% of this group. They are 13 and 12 amino acid residues long, respectively. Their sequences are identical to the N-terminal sequences of Lebetins 2. As observed for Lebetins 2, Lebetins 1 α and β differ by only a single Gly residue. These isoforms could perhaps be separated by anionic exchange chromatography.

Lebetins 1 might derive from Lebetins 2 by enzymatic cleavage. The nature of the processing and of the enzymes possibly involved needs to be delineated. The existence of a high molecular weight anti-aggregating precursor cannot be ruled out since Sephadex G-75 fraction II of the venom contained other antiplatelet aggregation compounds. These points are currently being investigated using antibodies directed against Lebetins 1 and Lebetins 2.

Lebetins 1 and Lebetins 2 inhibited rabbit and human platelet aggregation induced by various agonists. Lebetins 2 were generally more active than Lebetins 1. The anti-aggregating activities of Lebetins are similar to those of snake RGD disintegrins [16–19] and tick non-RGD disaggregin [20], the IC_{50}s of which range between 30 and 300 nM. However, Lebetins do not share significant sequence homology with these molecules or with any known protein. Lebetins are proline and lysine rich peptides and the tetrapeptide PPKK (residues 5–8) sequence is identical to residues 109–112 of a derived cow κ-casein undecapeptide [21], which inhibits ADP-induced platelet aggregation with an IC_{50} of about 100 μM. We observed here that Lebetins interfere with fibrinogen-induced aggregation but their mode of action remains entirely unknown: indeed, Lebetin-derived synthetic peptides do not in-





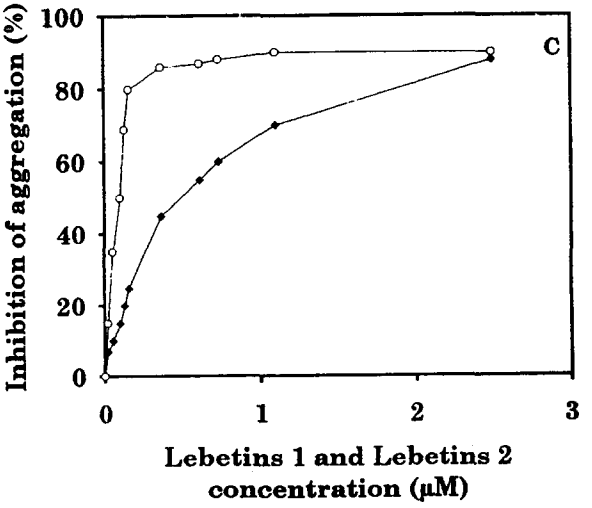


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Fig. 3. Inhibition of platelet aggregation in vitro by Lebetins. Rabbit or human platelets were incubated with Lebetins for 2 min at 37°C; agonists were then added (a representative experiment is shown for each condition; $n = 5$). A: Inhibition by Lebetins 1 of rabbit platelet aggregation induced by thrombin (■), PAF-acether (□), or collagen (●). B: Inhibition by Lebetins 2 of rabbit platelet aggregation induced by thrombin (■), PAF-acether (□), or collagen (●). C: Inhibition by Lebetins 1 (♦) and Lebetins 2 (○) of human platelet aggregation induced by thrombin.

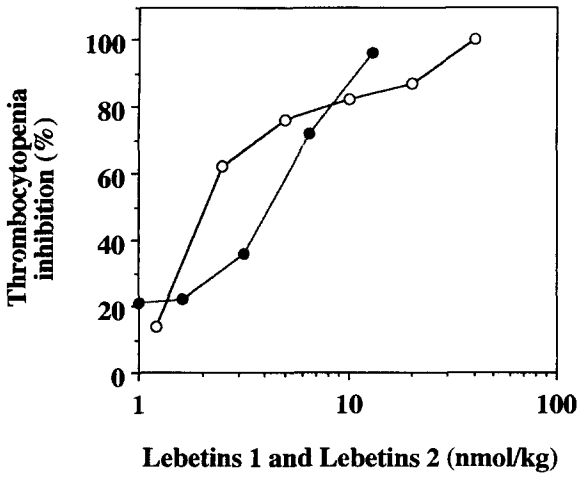

Fig. 4. Inhibition of the collagen-induced thrombocytopenia in rats by Lebetins. Lebetins 1 (○) or Lebetins 2 (●) were injected into the left jugular vein of anesthetized rats. After 2 min, collagen was injected into the jugular, and 1 min later, blood was sampled from the right carotid and platelet rich plasma prepared. The percentage of inhibition is the ratio of platelet counts in rats who received Lebetins versus those who received saline solution (a representative experiment is shown; $n = 5$).

terfere with fibrinogen-GPIIb/IIIa interaction per se as observed in a molecular assay (unpublished data).

As Lebetins are small non-toxic peptides at active doses and as the inhibition of the collagen-induced thrombocytopenia in rats by Lebetins is stronger than that obtained with the therapeutic synthetic compound SC 47643 [14], Lebetins could have the potency to be used as antithrombotic agents. It is worthy of note that, in contrast to in vitro, Lebetins 1 are about 2-fold more active than Lebetins 2 in vivo. A possible explanation of this result may be pharmacokinetic differences and/or a different antiplatelet effect between Lebetins 1 and Lebetins 2.

Finally, because they lack any known antiplatelet aggregation active sequence, these compounds might help to delineate still unknown mechanisms involved in platelet aggregation.

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