A platelet aggregation inhibitory protein, bitistatin, was isolated from the venom of the puff adder *Bitis arietans*. This protein is a single-chain peptide containing 83 amino acids and 7 disulfide bonds. Bitistatin contains the sequence arginine-glycine-aspartic acid and shows considerable homology to two previously described snake venom platelet aggregation inhibitors, trigramin and echistatin. Bitistatin inhibited human and canine platelet aggregation initiated by 10 μM ADP in vitro with IC₅₀ values of 2.37 ± 13 and 28 ± 3 μM, respectively. In order to assess the antithrombotic potential of bitistatin, a canine model of platelet-dependent coronary thrombus formation was utilized. Injection of bitistatin at 10–100 μg/kg (0.7–7 nmol/kg, intravenously (i.v.)) resulted in dose-dependent inhibition of both platelet aggregation ex vivo and platelet-dependent cyclical flow reductions. The effective dose to inhibit cyclical flow reductions was 30 μg/kg, i.v. A higher dose of bitistatin (100 μg/kg, i.v.) inhibited cyclical flow reductions for 160 ± 29 min as well as attenuated ex vivo platelet aggregation. Bitistatin at 100 μg/kg, i.v. prolonged the bleeding time 4 × normal at 15 min post-administration but returned to normal at 3 h. Thus, in a canine model of *in vivo* platelet aggregation, bitistatin is an effective antiplatelet agent to inhibit periodic cyclical flow reductions. Bitistatin also exhibits reversible effects of ex vivo platelet aggregation as well as on bleeding time.

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**Experimental Procedures**

**Purification of Bitistatin**—Canine platelet aggregation in vitro assays, employing 10 μM ADP and 1 μM epinephrine, were used to monitor activity during purification of bitistatin. A 5-μl aliquot of the protein fraction of interest was incubated with canine PRP for at least 1 min at 37 ℃ prior to the addition of agonists for the evaluation of platelet aggregation.

In order to obtain sufficient quantities of bitistatin for *in vivo* evaluation, a 30-μl clot of crude *B. arietans* venom was processed. Five-g portions of this venom (4000 mg of protein) were resuspended directly in 200 ml of 0.1% aqueous trifluoroacetic acid at room temperature. After 15 min, insoluble material was removed by centrifugation at 20,000 × g for 30 min. The crude solubilized venom caused platelet aggregation when added to canine PRP in the absence of agonists, preventing the detection of inhibitory activity in this fraction. C18 chromatography resolved the proaggregatory activity from aggregation inhibitory activity, allowing the latter to be detected. The solubilized venom was applied to a 5 × 30-cm Delta Pak C18 reverse-phase column and chromatographed on a Waters Delta Prep
Antithrombotic Effects of Bitistatin

FIG. 1. Fractionation of B. arietans crude venom. Crude venom (5 g) was dissolved in 200 ml of 0.1% trifluoroacetic acid. Following removal of insoluble material, the venom was loaded onto a 5 x 30-cm Delta Pak C18 reverse-phase column. The column was washed for 5 min with 0.1% trifluoroacetic acid, then eluted with a 0-65% acetonitrile linear gradient on 0.1% trifluoroacetic acid over 65 min. The flow rate was 100 ml/min. Fractions of 5 min each were collected. Fraction 6 contained the majority of the recovered platelet aggregation inhibitory activity. The solid line represents absorbance at 254 nm.

Amino Acid Analysis—Reduced and carboxymethylated peptides were hydrolyzed in vacuo for 20 h at 110 °C in the presence of 6 N HCl. The amino acid composition was then determined using a Beckman 6300 amino acid analyzer.

FIG. 2. Purification of bitistatin. Fraction 6 (see Fig. 1) was dissolved in 0.1% trifluoroacetic acid and applied to a 5 x 30-cm Delta Pak C18 reverse-phase column. The column was eluted with a 0-30% acetonitrile gradient on 0.1% trifluoroacetic acid over 60 min. The flow rate was 100 ml/min. Peaks 1-3 were pooled separately and tested for platelet aggregation inhibitory activity. Peak 1 was inactive. Peak 2, though active, was not further characterized. Peak 3 contained the majority of the recovered activity. This peak, called bitistatin, was employed in all subsequent studies.
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RESULTS

Purification and Characterization of Bitistatin—Bitistatin was purified from the soluble portion of the lyophilized venom of *B. arietans* by repetitive preparative scale C18 reverse-phase chromatography (Figs. 1 and 2). The purified protein was homogeneous as judged by analytical reverse-phase HPLC, SDS-polyacrylamide gel electrophoresis, and NH2-terminal sequence analysis. The native protein migrated with an apparent molecular mass of about 14,000 daltons during SDS-polyacrylamide gel electrophoresis. During gel filtration on Superose 12, the native protein exhibited an apparent mass of 8,600 daltons.

**Amino Acid Composition of Bitistatin**—The amino acid composition of bitistatin, obtained after 20 h of hydrolysis of the reduced carboxymethylated protein, is shown in Table I. This composition was compared with that predicted by the amino acid sequence analysis (see below). Most values obtained by these two methods agreed closely (Table I).

**Primary Structure of Bitistatin**—The primary structure of bitistatin was determined by automated sequence analysis (Fig. 4). Bitistatin contains 83 amino acid residues and has a calculated mass of 9022 daltons. The protein contains 14 cysteine residues. Sulphydryl titration of native bitistatin suggested that all cysteine residues are disulfide-bonded (data not shown). The disulfide bonding pattern is not known. Reduction and carboxymethylthiolation of these cysteine residues reduced the inhibitory potency by 60-fold.

The tripeptide sequence RGD is found at positions 64-66 of bitistatin. This sequence occurs twice in the Aa chain of fibrinogen (12) and is believed to mediate in part the binding of fibrinogen to platelet GP Ib-IIIa (11). Recently, the amino acid sequences of two RGD-containing viper venom-derived platelet aggregation inhibitors, trigramin (23) and echistatin (24), have been reported. Alignment of the sequences of these three proteins is shown in Fig. 5. The sequences of trigramin and echistatin are 68 and 49% identical to bitistatin, respectively.

**Effect of Bitistatin on Platelet Aggregation in Vitro**—Bitistatin was assessed *in vitro* in human and canine PRP for inhibition of platelet aggregation in response to a standard concentration of ADP (10 μM). Fig. 6 represents the percent

![CIRCUMFLEX BLOOD FLOW (ml/min)](image)

**FIG. 3.** Technique for monitoring platelet aggregation in the partially obstructed left circumflex coronary artery of the dog. Electromagnetic flow probes are utilized to measure blood flow in ml/min. Partial obstruction of the coronary artery with a plastic Lexan cylinder results in episodic cyclical reductions in coronary blood flow that are due to platelet-dependent thrombus formation. Every 2-3 min the thrombus must be mechanically shaken loose (SL) to restore blood flow.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/mol</th>
<th>Acid hydrolysis</th>
<th>Sequence</th>
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<tr>
<td>Alanine</td>
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<tr>
<td>Arginine</td>
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<tr>
<td>Glycine</td>
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<td>Glutamic acid*</td>
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<tr>
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<tr>
<td>Total</td>
<td>80.5</td>
<td>83</td>
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* The value includes asparagine.
* Cysteine was detected as the S-carboxymethylated derivative.
* The value includes glutamine.
* Tryptophan is destroyed by acid hydrolysis.
antithrombotic potential of bitistatin. The concentration of bitistatin that suppressed ADP-induced platelet aggregation by 50% was determined. The IC50 values for bitistatin in human and canine PRP, respectively, were 237 ± 13 and 28 ± 3 nM.

Effect of Bitistatin in Vivo—In order to determine the antithrombotic potential of bitistatin in vivo, an animal model of periodic platelet aggregation in the circumflex coronary artery was utilized. Bitistatin was injected as an i.v. bolus in 2 ml of saline at doses of 10, 30, and 100 μg/kg. Fig. 7 illustrates the dose-dependent inhibitory effect of bitistatin on CFRs in the circumflex coronary artery of the dog. The top panel of Fig. 7 represents control aggregation in the coronary artery in which every 2–3 min coronary flow reached zero and the thrombus was mechanically dislodged by shaking (SL) the cylinder. Injection of bitistatin at 10 μg/kg, i.v. (Fig. 7, second panel) lengthened the time to complete occlusion but did not prevent periodic thrombus formation. However, bitistatin at a bolus dose of 30 μg/kg, i.v. (Fig. 7, third panel) was an effective antithrombotic and antiaggregatory dose as evidenced by complete restoration of coronary blood flow in which aggregates were spontaneously (X) shed from the obstructed lumen without the need for mechanical dislodgement. At this dose of bitistatin small aggregates continued to form but were shed spontaneously before flow approached zero. Bolus injection of bitistatin at 100 μg/kg, i.v. (Fig. 7, fourth panel) completely inhibited platelet aggregation in the coronary artery as flow was maintained at a very steady level without aggregate formation. Thus, 30 μg/kg, i.v. bitistatin was an effective antithrombotic dose whereas 100 μg/kg, i.v. bitistatin produced a maximal antithrombotic effect.

The duration of the antiaggregatory effect of bitistatin at 30 and 100 μg/kg, i.v. was determined (Table II). Duration of effect was calculated from the time of the initial inhibitory effect of thrombus formation to the time at which coronary flow was again at zero. Bitistatin at 30 and 100 μg/kg, i.v. maintained coronary blood and prevented flow from approaching zero for 103 ± 26 and 160 ± 29 min, respectively. The effect of bitistatin on ex vivo platelet aggregation in response to ADP was also determined in this study coincident with the in vivo analysis of CFRs. Blood was withdrawn from the dog before and after injection of bitistatin, and platelet-rich plasma (300,000 platelets/μl) was prepared. Bitistatin at 30 μg/kg, i.v. inhibited platelet aggregation in response to 5 μM ADP and 10 μM ADP 15 min following administration by 17 and 24%, respectively (Table III). One hour following bitistatin at 30 μg/kg, i.v., ADP-induced ex vivo platelet aggregation was still inhibited to the same degree and CFRs in the coronary artery remained abolished. Two hours following administration of bitistatin at 30 μg/kg, i.v., ex vivo platelet aggregation to ADP was restored to control levels and CFRs had resumed in the coronary artery. Thus, there was a clear correlation between inhibition of platelet aggregation ex vivo in response to ADP and in vivo as assessed by CFRs.

At a bitistatin dose of 100 μg/kg, i.v., inhibition of ex vivo platelet aggregation in response to ADP was greater than that observed at 30 μg/kg. ADP-induced platelet aggregation 15 min following bitistatin at 100 μg/kg was reduced by greater than 50% (Table III). Aggregation returned to control levels 3 h following administration of bitistatin at 100 μg/kg, i.v. which coincided with restoration of CFRs in the coronary artery (Table II). Therefore, bitistatin at a bolus dose of 100 μg/kg, i.v. resulted in inhibition of platelet aggregation both ex vivo and in vivo for almost 3 h.

Ex vivo platelet aggregatory responses to the endoperoxide/thromboxane A2 mimetic U-46619 and to collagen were also determined before and after administration of bitistatin (100 μg/kg, i.v.) to the dog. Fig. 8 illustrates that bitistatin attenuated U-46619 and collagen-induced platelet aggregation to a similar degree as determined previously with ADP.

We also assessed the prolongation of the bleeding time in response to bitistatin injections. At 100 μg/kg, i.v. bitistatin prolonged the bleeding time from 2.8 to 13.6 min, 15 min following administration (Table IV). This represents a 4.3-fold increase in the bleeding time over control (Table V). However, 3 h following administration of this dose (100 μg/kg, i.v.), the bleeding time was restored to normal (Table V). The change in bleeding time for bitistatin at 30 μg/kg i.v.
increased from 2.8 to 5.6 min or 2-fold (Table IV). Bitistatin at 10 μg/kg, i.v. had little or no effect on bleeding time (Table IV).

**DISCUSSION**

The results of this study indicate that the RGD-containing peptide bitistatin is a potent inhibitor of platelet aggregation assessed in vitro, ex vivo, and in vivo in a canine model of repetitive thrombus formation. Bitistatin displayed dose-responsive antithrombotic effects with 10 μg/kg, i.v. being a threshold anti-aggregatory dose and 100 μg/kg, i.v. exhibiting full inhibition of platelet aggregation. The high dose of bitistatin (100 μg/kg, i.v. bolus) inhibited platelet aggregation in the coronary artery for almost 3 h with a concomitant inhibition of ex vivo platelet aggregation and an increase (4–5-fold) in the bleeding time. Three hours following 100 μg/kg bitistatin, ex vivo platelet aggregation and bleeding time had returned to control levels. Thus, bitistatin is a reversible inhibitor of platelet aggregation in the dog and can be administered by a single i.v. bolus injection.

Bitistatin is a small stable protein which is relatively abundant in the commercially available venom of the puff adder *B. arietans*. It is clear from consideration of the primary structure of bitistatin (Fig. 5) that it is part of a family of viper venom-derived platelet aggregation inhibitor proteins, which also includes trigramin and echistatin.

We chose *B. arietans* venom as a potential source of platelet aggregation inhibitors based upon a previous report by Joubert et al. (32). These workers described the isolation of a protein called CM-2 from the venom of *B. arietans*. This protein exhibited a considerable sequence homology to both trigramin and echistatin, but its platelet aggregation inhibitory activity was not evaluated. Of special interest was the fact that CM-2 was reported to have the sequence arginine-glycine-asparagine (RGN) in place of the RGD sequence of trigramin and echistatin. Thus we wondered whether an RGN-containing protein could have platelet aggregation-inhibiting properties. This question remains unanswered at present since the protein we isolated, bitistatin, while showing considerable NH₂-terminal homology to CM-2, did in fact contain the sequence RGD rather than RGN (Fig. 4).

Numerous antithrombotic approaches have been evaluated experimentally and clinically. One of the first agents widely utilized was aspirin which irreversibly blocks the cyclooxygenase enzyme, thus inhibiting the synthesis not only of proaggregatory thromboxane A₂ but also of antiaggregatory prostanooids such as PGD₂ and PGL₁ (33, 34). In a search for more selective agents, thromboxane synthase inhibitors were developed such that thromboxane A₂ synthesis was blocked but synthesis of antiaggregatory prostaglandins was unaffected (35). Extensive evaluation of such inhibitors showed that although thromboxane synthesis was prevented, prostaglandin endoperoxides could still aggregate platelets in some cases (36). Therefore, thromboxane receptor antagonists capable of
The results of this study indicate that RGD-containing peptides such as bitistatin may be valuable antithrombotic agents. Not only do agents of this class (i.e., fibrinogen receptor antagonists) block platelet aggregation independent of the nature of the platelet agonists, but the effects are dose-dependent and reversible which allows for careful clinical titration of the dose. Agents of this type may be antithrombotic in their own right but may also influence thrombolysis in a positive manner by increasing the rate of fibrinolysis and preventing reocclusion. Studies of the effects of fibrinogen receptor antagonists administered in conjunction with thrombolytic agents are currently in progress.

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