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RESEARCH PAPER

Antithrombotic effect of Z4A5 on coronary thrombosis in a canine model of acute unstable angina

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BACKGROUND AND PURPOSE

The glycoprotein IIb/IIIa receptor is the final common pathway of platelet aggregation, regardless of the agonist, and thus represents an ideal therapeutic target for blocking coronary thrombosis. In this study, the anti-platelet and antithrombotic actions of Z4A5, a new glycoprotein IIb/IIIa receptor inhibitor, were evaluated in a canine model of acute unstable angina.

EXPERIMENTAL APPROACH

Z4A5 was given i.v. as a bolus followed by 60 min of continuous infusion at doses of 30 $\mu g \cdot kg^{-1}$ + 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 30 $\mu g \cdot kg^{-1}$ + 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ or 300 $\mu g \cdot kg^{-1}$ + 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$. Its antithrombotic effect was evaluated in a model of coronary thrombosis, the injured, stenosed left circumflex coronary artery, in which platelet-dependent cyclic flow reductions (CFRs) were induced by vascular compression and constriction to simulate clinical acute unstable angina. Platelet aggregation and coagulation parameters were determined in platelet-rich plasma and platelet poor plasma respectively.

KEY RESULTS

The Z4A5 infusion induced a dose-dependent reduction in CFR frequency, which returned to baseline levels after the termination of the infusion at low doses. At medium dose that inhibited most part of platelet aggregation, it increased tongue bleeding time marginally with no dramatic changes in haemodynamic and coagulation parameters. Furthermore, the inhibition of ADP-induced platelet aggregation and prolonged bleeding time observed during Z4A5 infusion reverted to baseline levels after the termination of the infusion.

CONCLUSIONS AND IMPLICATIONS

Z4A5 is an effective antithrombotic agent for coronary artery thrombosis with a rapid-on and rapid-off pharmacological profile, and could be used as an alternative treatment of coronary artery ischaemic syndromes.

Abbreviations

AA, arachidonic acid; APTT, activated partial thromboplastin time; ASC, acute coronary syndromes; CFR, cyclic flow reduction; GP, glycoprotein; GPIs, glycoprotein IIb/IIIa receptor inhibitors; LCX, left circumflex; LSD, least-significant difference; NS, normal saline; NSTEMI, non-ST-elevation myocardial infarction; PCI, percutaneous coronary intervention; PPP, platelet poor plasma; PRP, platelet-rich plasma; PT, prothrombin time; RGD, Arg-Gly-Asp; STEMI, ST-segment elevation myocardial infarction; TH, thrombin; UA, unstable angina

Introduction

While platelets play a critical role in preventing blood loss in response to injury, they are also involved in the formation of pathogenic thrombi that cause the symptoms of vascular thrombotic disease, such as acute coronary syndromes (ACS) and acute ischaemic complications of percutaneous coronary intervention (PCI) (Priomos, 2001; Jennings, 2009). Antiplatelet therapies that block platelet activation and aggregation have established clinical benefits for patients presenting with acute cardiovascular events. The glycoprotein IIb/IIIa receptor is the final common pathway of platelet aggregation, regardless of the agonist, and thus represents an ideal therapeutic target for blocking coronary thrombosis (Phillips *et al.*, 1991; Jackson *et al.*, 2003). Multiple randomized clinical trials have shown that i.v. platelet glycoprotein IIb/IIIa receptor inhibitors (GPIs) significantly reduce the risk of death or reinfarction in patients with non-ST-elevation myocardial infarction (NSTEMI) (Kong *et al.*, 1998; Speich *et al.*, 2009). According to guidelines, GPIs are the most potent antiplatelet agents adopted as an adjuvant treatment during PCI in patients with ST segment elevation myocardial infarction (STEMI) (Hamm *et al.*, 1999; Wang *et al.*, 2012).

The clinical effectiveness of platelet glycoprotein IIb/IIIa (GP IIb/IIIa) antagonists has been attributed to the prevention of fibrinogen-mediated aggregation of activated platelets (Bennett, 2001). The presence of the Arg-Gly-Asp (RGD) tripeptide sequence on each fibrinogen molecule allows it to act as a multivalent ligand, cross-linking activated platelets during the aggregation process (Plow *et al.*, 1985; Lynch *et al.*, 1995). Antagonism of the fibrinogen platelet GPIs by targeting the RGD recognition sequence therefore represents an attractive antithrombotic mechanism for potential use in the treatment of vascular occlusive disorders.

Z4A5, a new platelet GP IIb/IIIa receptor antagonist, is a linear peptide with double RGD recognition sequences (Li *et al.*, 2012). This compound was synthesized as an analogue of a previously identified series of GP IIb/IIIa antagonists designed as a mimetic of the α-chain of fibrinogen. In our previous studies, Zhou *et al.* (2011) demonstrated that platelet-targeted microbubbles combined with Z4A5 were beneficial in preventing re-thrombosis in a canine model of femoral artery endothelial injury. Moreover, Z4A5, alone or in combination with aspirin and/or heparin, has recently been shown to have antithrombotic activity in a rabbit model of arteriovenous shunt thrombosis (Jing *et al.*, 2011). However, the antithrombotic activity of Z4A5 on coronary events has not been investigated until now. In the present study, we demonstrated that Z4A5 has antithrombotic and anti-platelet activity both the *in vitro* and *in vivo*. The antithrombotic efficacy of Z4A5 was assessed in canine models of coronary artery thrombosis in which platelet-dependent cyclic flow reductions (CFRs) were induced by vascular compression and constriction to simulate clinical acute unstable angina.

Methods

Preparation of animals

Adult New Zealand rabbits (1.5–2.5 kg) of either sex and Chinese rural dogs (9.0–11.0 kg) were purchased from the Experimental Animal Centre of Xi'an Jiaotong University, China. The animals were kept in a temperature-controlled environment (25 ± 1°C) with a 12 h/12 h light/dark cycle and fed standard chow for at least 1 week before any manipulation. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The animals were kept and treated in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care. The study was approved by the Ethical Committee of Xi'an Jiaotong University.

Canine model of coronary artery CFRs

Platelet-dependent CFRs were induced in the left circumflex (LCX) coronary artery of anaesthetized dogs with a modification of the method described by Bertha and Folts (1984). Dogs were anaesthetized with sodium pentobarbital (30 mg·kg^{-1}, i.v.) and ventilated with room air with a respirator pump (V10B, Gene & I Scientific Ltd, Beijing, China). Body temperature was maintained at 37°C with a heating table. Catheters were placed in a femoral vein and artery for drug administration and blood sampling respectively. Arterial blood pressure and heart rate were continuously recorded with a standard limb lead electrocardiograph (RM-6000, Nihon Kohden Corporation, Tokyo, Japan). The heart was suspended in a pericardial cradle through a left thoracotomy in the fifth intercostal space, and the left circumflex (LCX) coronary artery was isolated proximal to the first marginal branch. An electromagnetic flow probe (RM-6000, Nihon Kohden Corporation, Tokyo, Japan) was placed on the LCX coronary artery, and blood flow was monitored continuously. All physiological parameters were recorded on a data acquisition system (Powerlab/8sp, ADInstruments, Australia).

After a 30 min stabilization period, the proximal dissection site of the LCX coronary artery was injured by applying three occlusions for 10 s with a 3 mm interval with spring-loaded forceps. A metal constrictor with a flexible shaft, 2 mm in length, designed to produce a 60% to 80% narrowing of the vessel, was then placed in the middle of the injured site. A gradual decline in blood flow due to platelet adhesion and aggregation was observed. Critical coronary artery stenosis was achieved until the reactive hyperaemic response to a temporary occlusion was nearly abolished. This spontaneous repetitive pattern of decreasing blood flow following mechanical restoration resulting from the combination of endothelial damage and critical stenosis is referred to as CFR. The number of CFRs is determined by the number of times the thrombus needed to be dislodged.

Femoral arterial thrombosis induced by ferric chloride

This model was adapted from a similar injury model in rats (Kurz *et al.*, 1990). Dogs were anaesthetized with sodium pentobarbital (30 mg·kg^{-1}). A 2–3 cm segment of femoral artery was gently isolated from surrounding tissue, and an electromagnetic flow probe (RM-6000, Nihon Kohden Corporation, Tokyo, Japan) was placed around the distal dissection site. A filter paper disk (diameter 1 cm) saturated with 30% $FeCl_3$ solution was placed on the surface of the artery for

40 min. The artery was isolated immediately after removing the disk and opened lengthwise. The thrombus was scraped out, and its wet weight was measured immediately. The dry weight was determined after drying at room temperature for 24 h. Z4A5 or a control solution was administered i.v. 10 min before the application of ferric chloride. Reduced blood flow was recorded.

Blood collection

In vitro *experiments*. Human venous blood was obtained from 30 healthy volunteers (20–40 years old, 15 male and 15 female) who had not taken any drugs known to affect platelet function for 2 weeks before the study. The experiments were conducted with the understanding and the consent of each participant. Approval for experiments with human blood was provided by the Human Ethics Committee of Xi'an Jiaotong University.

After the animals were anaesthetized with sodium pentobarbital (30 mg·kg^{-1}, i.v.), fresh blood was collected from the rabbits' carotid arteries and the dogs' femoral arteries.

Ex vivo *experiments*. Dogs were anaesthetized with sodium pentobarbital (30 mg·kg^{-1}, i.v.), and a catheter was placed in a femoral artery for blood sampling. Blood samples (3 mL) were obtained at 0 min (before control or drug administration); at 5, 15, 30 and 60 min during the infusion; and at 15, 30 and 60 min after the termination of the infusion.

Preparation of platelet-rich plasma (PRP)

The samples used for the determination of platelet aggregation and coagulation parameters were prepared according to standard practice (Bengmark *et al.*, 1981). Blood was collected into a plastic syringe containing one part 3.8% trisodium citrate to nine parts blood. PRP was collected following centrifugation at 100× *g* for 10 min at room temperature. Platelet poor plasma (PPP) was prepared from the same blood sample by further centrifugation at 800× *g* for 10 min at room temperature. PRP was diluted to 2.0 × 10^8 platelets·mL^{-1} with antologous PPP.

Platelet aggregation

Platelet aggregation was assessed at 37°C with a four-channel platelet aggregation analyser (LBY-NJ4, Beijing Precil Instrument Co. Ltd, China) by recording increased light transmission through a stirred suspension of PRP. Platelet aggregation is expressed as a percentage of light transmittance with PPP representing 100% transmittance. Aggregation curves were recorded for 5 min, and the value of maximal light transmission was used to calculate the aggregation percentage.

For *in vitro* experiments, PRP samples (285 μL) were first incubated with stirring for 5 min at 37°C in the presence of placebo or Z4A5 and then with 5 μL of platelet agonists (ADP, 20 μmol·L^{-1}; arachidonic acid AA, 10 mmol·L^{-1}; and thrombin, 200 U·L^{-1}) for 5 min at 37°C. A sub-aggregatory dose of adrenaline (5 μL, 1 μmol·L^{-1}) was used to strengthen the platelet aggregation immediately after the addition of agonists. The percentage inhibition was calculated based on the maximum aggregation rate of the test samples relative to the appropriate buffer control.

For *ex vivo* experiments, PRP was prewarmed for 5 min at 37°C before the addition of the agonist ADP (20 μM in the presence of 1 μM adrenaline) to the cuvette. The percentage inhibition was calculated based on the maximum aggregation rate at the different times during or after the administration of the drug or control and the pre-administration rate.

Coagulation parameters

Coagulation parameters were evaluated immediately before initiating Z4A5 or control infusion (0 min); at 5, 15, 30 and 60 min during infusion; and at 15, 30 and 60 min after the termination of infusion. PPP was obtained for analysis by a method similar to that described above. Plasma coagulation was assessed by determining prothrombin time (PT, PT Kit, Steellex Scientific Instrument Company, China) and activated partial thromboplastin time (APTT, APTT Kit, Steellex Scientific Instrument Company, China) with a Coagulation Analyser (LG-PABER-I, Steellex Scientific Instrument Company, China).

Tongue-template bleeding time

Bleeding times were determined at baseline (0 min), 5, 15, 30 and 60 min during drug infusion; and at 15, 30 and 60 min after the termination of infusion. An automated incision device (KJ119, Jiangsu Kangjian Medical Apparatus Co. Ltd, China) was used to make a standardized incision 5 mm long and 1 mm deep on the upper surface of the tongue. The lesion was carefully blotted with filter paper every 20 s until bleeding stopped.

Experimental protocols

In vitro *experiments*. Z4A5 was dissolved in 0.9% saline to obtain 1 mmol·L^{-1} primary solutions. The primary solutions were serially diluted with 0.9% saline to obtain appropriate concentrations. A 0.9% saline solution was used as the control. Five groups were tested in this protocol.

1 Human (n = 10), induced by ADP, Z4A5 concentration 1500–120 nmol·L^{-1}
2 Human (n = 10), induced by AA, Z4A5 concentration 500–41 nmol·L^{-1}
3 Human (n = 10), induced by TH, Z4A5 concentration 720–59 nmol·L^{-1}
4 Rabbit (n = 8), induced by ADP, Z4A5 concentration 400–3.1 μmol·L^{-1}
5 Dog (n = 6), induced by ADP, Z4A5 concentration 1000–7.8 nmol·L^{-1}

Ex vivo *experiments*. The experimental protocol is shown in Figure 1. After a 60 min control period of reproducible CFRs (time = –60 min to 0 min), test agents (saline or Z4A5) were given i.v. as a loading bolus (2 mL) followed by a continuous infusion (60 mL) for 60 min (time = +60 min). Monitoring was continued for 60 min after the termination of drug infusion (time = +120 min). The antithrombotic effect was quantified by comparing the number of CFRs per hour before, during and after drug infusion. Femoral arterial thrombosis was induced by applying ferric chloride during the infusion from 10 min to 50 min. Four groups were tested in this protocol.

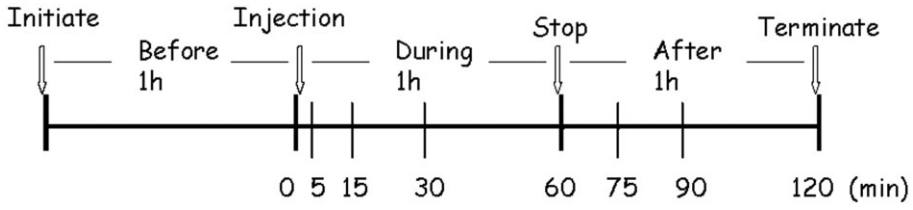

Figure 1

Experimental protocol for the canine model of LCX coronary artery CFRs. The antithrombotic effect was quantified by comparing the number of CFRs per hour before, during and after drug infusion. Blood samples were obtained at 0, 5, 15, 30, 60, 75, 90 and 120 min during Z4A5 or control infusion.

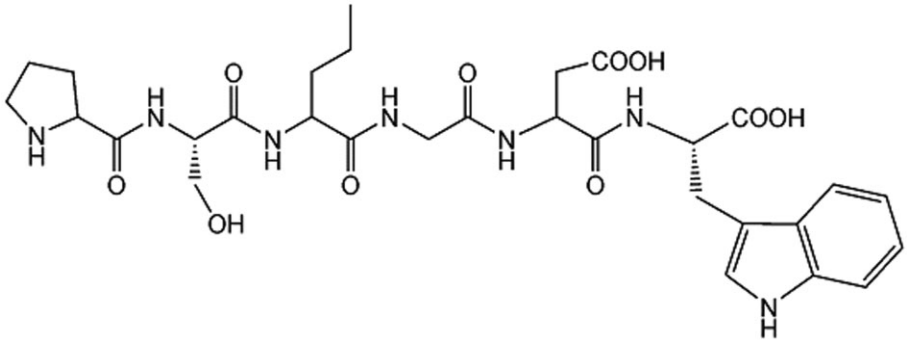

Figure 2

The chemical structure of Z4A5. The hex-peptide amino acid sequence: Pro-Ser-Nva-Gly-Asp-Trp. Z4A5 was dissolved in 0.9% sodium chloride solution for injection.

1 Control (physiological saline, $n = 6$).
2 Low dose Z4A5 (30 $\mu g \cdot kg^{-1}$ + 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$, $n = 6$).
3 Middle dose Z4A5 (30 $\mu g \cdot kg^{-1}$ + 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$, $n = 6$).
4 High dose Z4A5 (300 $\mu g \cdot kg^{-1}$ + 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$, $n = 6$).

Drugs

The hex-peptide Z4A5 was synthesized by solid-phase peptide synthesis methods with standard functionality–protected amino acids. Compounds were purified to 97% purity by HPLC and characterized by NMR, mass spectrometry and amino-acid analysis. The chemical structure of Z4A5 is shown in Figure 2. Z4A5 was dissolved in 0.9% sodium chloride solution for injection.

Statistical analysis

All values are expressed as the mean ± SEM. Data were analysed with the SPSS 11.5 software package for Windows. All variable of distributions were tested for normality with the Kolmogorov–Smirnov test. Group differences for *in vitro* platelet aggregation percentage inhibition, thrombus weight and reduction in femoral artery blood flow were performed by one-way ANOVA followed by least-significant difference (LSD) *post hoc* tests. The IC_{50}s for Z4A5 inhibition of ADP, AA or TH-induced platelet aggregation were calculated with Probit Regression. Changes in mean arterial pressure, heart rate, CFR frequency, *ex vivo* platelet aggregation induced by ADP, coagulation parameters and bleeding time during the experiment were analysed by repeated measure one-way ANOVA followed by LSD *post hoc* tests. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of Z4A5 on human platelet aggregation in vitro

Z4A5 dose-dependently inhibited human platelet aggregation *in vitro* at concentrations ranging from 1.5×10^{-6} to 4.1×10^{-8} $mol \cdot L^{-1}$ ($P < 0.05$). Z4A5 suppressed AA-induced platelet aggregation at low concentrations with an IC_{50} value of approximately 173 nM (95% confidence limits: 94–227 nM, Figure 3B). The IC_{50} value for TH-induced aggregation was approximately 205 nM (95% confidence limits: 183–226 nM, Figure 3C). The inhibition of ADP-induced aggregation was relatively weak (IC_{50} = 451 nM, 95% confidence limits: 400–498 nM, Figure 3A).

Effect of Z4A5 on animal platelet aggregation induced by ADP in vitro

Z4A5 significantly inhibited rabbit platelet aggregation induced by ADP *in vitro* in a concentration-dependent

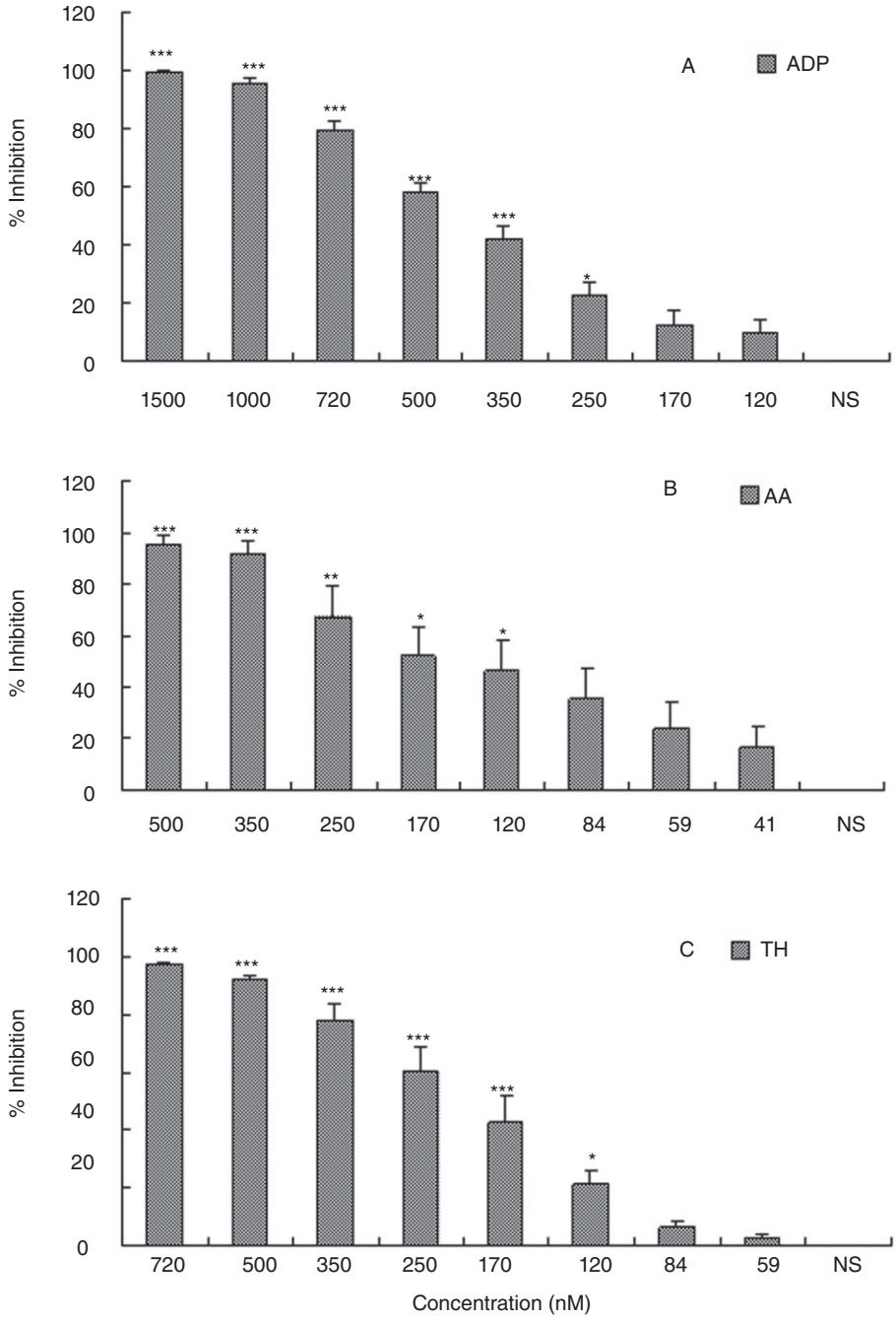

Figure 3

Effect of increasing concentrations of Z4A5 on human platelet aggregation *in vitro*. (A) ADP-induced, (B) AA-induced, (C) TH-induced aggregation. Data are the mean ± SEM, $n = 10$ for each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control (NS).

manner ($P < 0.05$). Complete inhibition was obtained at 400 μM with an IC_{50} of 38.71 μM (95% confidence limits: 32.38–44.93 μM) in the presence of 20 μM ADP (Figure 4A). Z4A5 also significantly ($P < 0.05$) and concentration-dependently suppressed ADP-induced dog platelet aggregation *in vitro*, but at lower concentrations, with an IC_{50} value of approximately 87.20 nM (95% confidence limits: 70.20–104.15 nM, Figure 4B).

Haemodynamic effects

The haemodynamic effects of i.v. administration of Z4A5 are summarized in Table 1. The mean arterial pressure and heart rate did not differ significantly between groups of dogs under baseline conditions ($P > 0.05$). The i.v. administration of either Z4A5 or the control (0.9% sodium chloride solution) did not result in significant differences in heart rate and

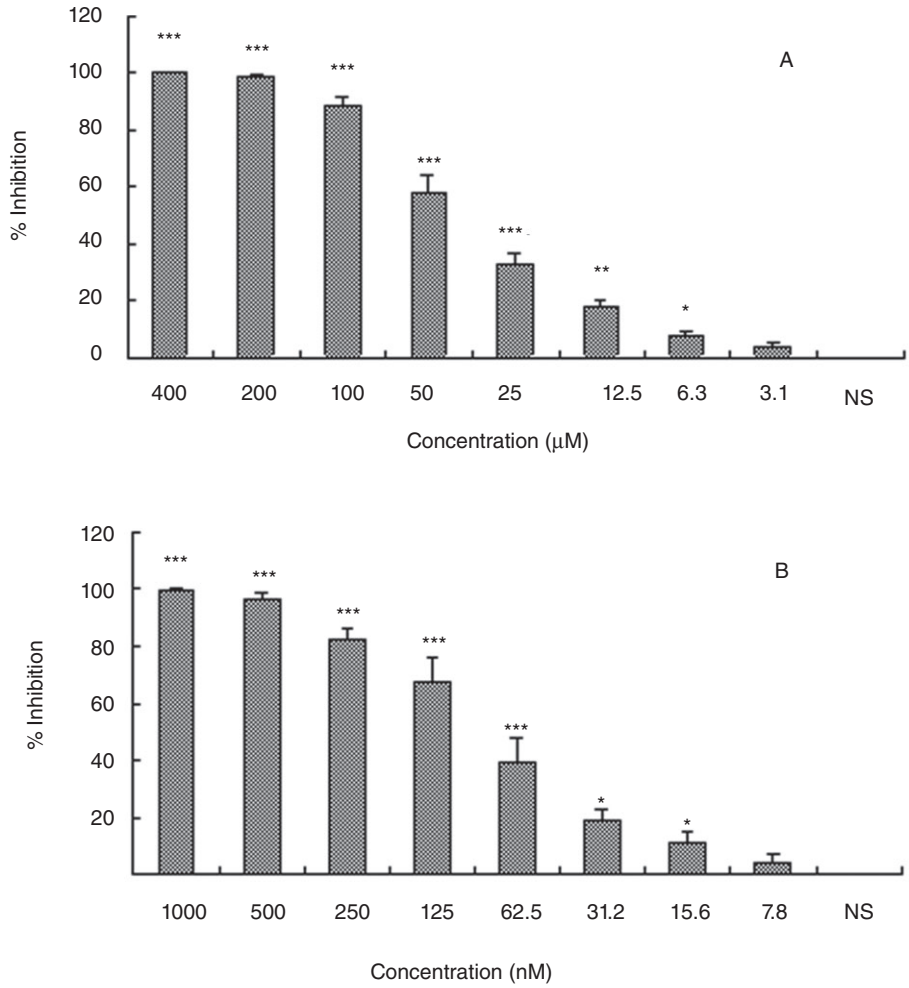

Figure 4

Effect of Z4A5 on ADP-induced animal platelet aggregation *in vitro*. Values are means ± SEM (A) Rabbit platelet aggregation ($n = 8$) and (B) dog platelet aggregation ($n = 10$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control (NS).

blood pressure compared to the respective controls during injury of the femoral or coronary artery ($P > 0.05$).

Canine model of coronary artery CFRs

Using a coronary artery CFR model, the antithrombotic efficacy of different doses of Z4A5 was tested in dogs. A representative CFR trace is shown in Figure 5A, and the effect of Z4A5 on CFR frequency is shown in Figure 5B. After saline administration, the CFR frequency was unaltered compared to the baseline ($P > 0.05$). At the lower dose of Z4A5 (30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}, $P < 0.05$), the number of CFRs was reduced by 39% compared with the baseline and returned to the baseline level after the termination of administration at 1 h. At the two higher doses of Z4A5 (30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} and 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}), CFRs were nearly completely abolished immediately after the loading bolus (>90% inhibition, $P < 0.01$) and returned to greater than 30% of baseline levels by 1 h after administration ($P < 0.01$).

Femoral arterial thrombosis

Z4A5 showed significant antithrombotic effects in the canine femoral arterial thrombosis model ($P < 0.05$, Figure 6). Compared with the negative control, Z4A5 decreased wet thrombus weight from 35.82 ± 9.76 mg to 16.62 ± 4.22, 7.62 ± 2.14 and 6.38 ± 3.76 mg at doses of 30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}, 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} and 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} respectively (Figure 6A). Meanwhile, dry thrombus weight decreased significantly 56%, 79% and 79% from the control values at the same doses (Figure 6B). Moreover, the reduction in femoral artery blood flow was prevented by Z4A5 in a dose-dependent fashion parallel to the reduction in thrombus weight (Figure 6C). In the ferric chloride model, blood flow was reduced by approximately 42% (41.68 ± 5.22%) in control animals. Z4A5 induced a dose-dependent inhibition of the blood flow reduction by 26.20 ± 7.42, 16.90 ± 3.25 and 8.53 ± 1.40%, at low, medium and high doses.

Platelet aggregation induced by ADP

Inhibition of *ex vivo* platelet aggregation in response to ADP was observed upon the continuous i.v. infusion of Z4A5 to anaesthetized dogs for 60 min (Figure 7A). Z4A5-treated dogs exhibited a sharp decrease in ADP-induced platelet aggregation after 5 min of infusion that remained at this level until

Table 1
Effects of Z4A5 and control solution on mean arterial pressure and heart rate in dogs

	n	Mean arterial pressure (mm Hg)					Heart rate (bpm)				
		Baseline	Before	During	After	*P*	Baseline	Before	During	After	*P*
Control	6	114.63 ± 4.02	92.76 ± 4.95	91.60 ± 5.29	100.06 ± 5.90	ns	151.74 ± 10.03	146.16 ± 12.42	144.46 ± 12.78	146.26 ± 14.37	ns
300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}	6	123.56 ± 4.73	116.73 ± 5.37	116.67 ± 3.57	122.09 ± 5.76	ns	186.09 ± 6.62	188.62 ± 6.97	190.12 ± 8.34	192.90 ± 9.65	ns
30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}	6	113.67 ± 9.62	102.18 ± 11.42	99.68 ± 11.02	103.79 ± 7.62	ns	167.34 ± 9.49	164.91 ± 12.42	165.65 ± 16.84	156.45 ± 16.46	ns
30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}	6	114.56 ± 7.01	103.99 ± 5.19	110.32 ± 8.36	109.63 ± 6.93	ns	157.15 ± 9.79	149.58 ± 8.54	151.09 ± 10.27	152.91 ± 11.01	ns

the end of the infusion, compared with untreated controls, which exhibited relatively constant aggregation ($P < 0.01$). The 30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1} dose of Z4A5 elicited minimal (50–60%) inhibition of ADP-induced aggregation, whereas the administration of 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} and 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} of Z4A5 maintained 80% to 90% inhibition of ADP-induced platelet aggregation, throughout the 60 min infusion. In all treatment groups, the inhibition of ADP-induced platelet aggregation declined to <30% by 60 min after the termination of Z4A5 infusion.

Coagulation parameters

Both prothrombin time (PT) and activated partial thromboplastin time (APTT) were consistent between treatment groups before the administration of Z4A5 ($P > 0.05$), and there were no statistically significant differences in PT and APTT among the four groups at any time during administration ($P > 0.05$). Even at a dose of 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}, Z4A5 caused no notable changes in PT or APTT compared to the control (PT, $P > 0.05$; APTT, $P > 0.05$). Thus, the coagulation parameters (PT and APTT) were not affected by Z4A5 (Table 2).

Bleeding time

The effect of Z4A5 on tongue bleeding time is shown in Figure 7B. We chose a maximum of 20 min for the measurement of the tongue bleeding time. If the lesion continued to bleed after 20 min, the measurement was stopped, and the bleeding time recorded at 20 min was used for statistical analysis. The lower dose of Z4A5 (30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}) tended to increase bleeding time; however, the change was not statistically significant compared with the control group ($P > 0.05$). During the infusion of 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}, the bleeding time increased by 2.4-, 2.7-, 3.0- and 2.1-fold at 5, 15, 30 and 60 min respectively. As expected, when the dose of Z4A5 was increased to 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}, the bleeding time increased significantly at 5, 15 and 30 min after the initiation of the drug infusion. However, in all Z4A5-treated groups, the tongue bleeding time had declined to the baseline level by 15 min after the termination of the Z4A5 infusion.

Discussion

Platelet aggregation is a key step in arterial thrombus formation. Z4A5 is a low-molecular-weight peptide that structurally mimics the RGD fibrinogen binding sequence in GP IIb/IIIa. In the present study, the antithrombotic effects of Z4A5 were evaluated in canine models of coronary and femoral arterial thrombosis. Our results demonstrate an *in vivo* antithrombotic effect of Z4A5 doses form 30 μg·kg^{-1} as a loading bolus followed by continuous infusion at 1 μg·kg^{-1}·min^{-1} to 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}, without any effect on the main coagulation parameters. Meanwhile, *ex vivo* ADP-induced platelet aggregation was inhibited by Z4A5 in a dose-dependent fashion in parallel with a reduction in thrombus weight.

Platelet-dependent thrombosis is an important part of the pathophysiology of acute coronary syndrome (ACS), includ-

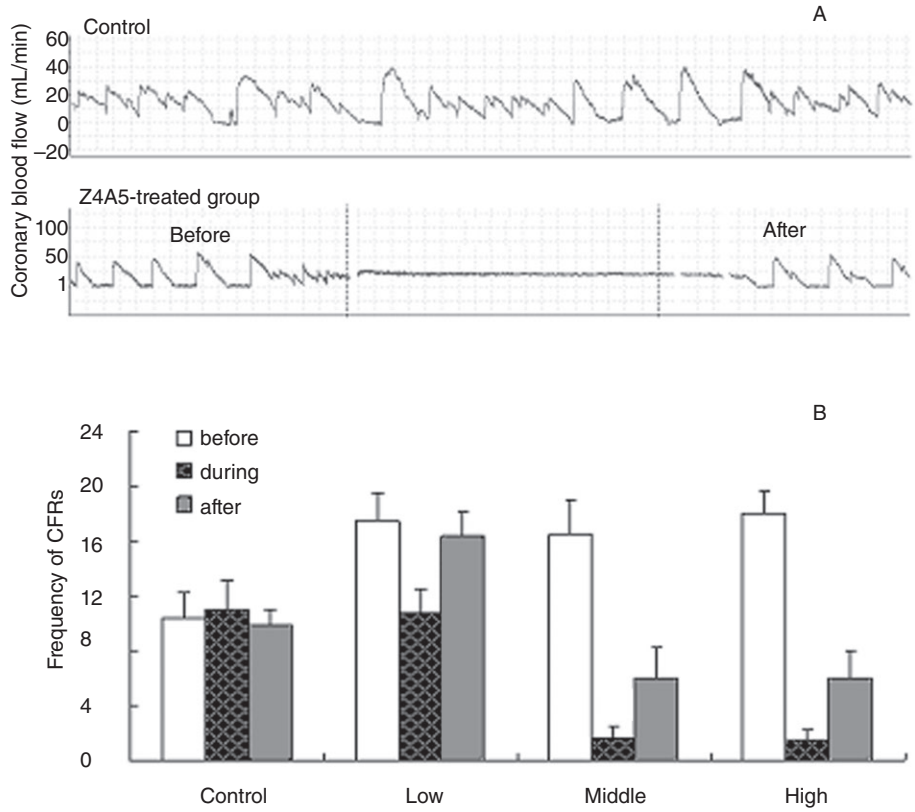

Figure 5

Effect of Z4A5 and control solution on CFRs in a canine model. Z4A5 was administered i.v. as a loading bolus followed by a continuous infusion for 60 min at dose of 30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}, 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} or 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}. Values are the means ± SEM for $n = 6$ experiments. (A) Representative recordings of cyclic flow reductions in blood flow in the left circumflex dog coronary artery caused by a critical stenosis and vessel damage. (B) The frequency of CFRs in the canine model in the absence and presence of Z4A5. *$P < 0.05$ versus control.

ing PCI (Braunwald, 1998). CFR has been demonstrated in human coronary arteries after angioplasty (Eichhorn *et al.*, 1991). CFR occurs via the repetitive accumulation of platelet aggregates at sites of coronary endothelial injury. The partial occlusion by the aggregates leads to a gradual decline in coronary blood flow and to the build-up of a pressure gradient across the aggregates. When the gradient reaches a certain threshold, the aggregates dislodge with a sudden restoration of coronary flow. CFR thus not only can be considered an indicator of *in situ* platelet activation but is also predictive of acute ischaemic complications during PCI, which can be eliminated by GP IIb/IIIa receptor antagonists (Anderson *et al.*, 1994; Kao *et al.*, 2002). Over the past decade, more than 30 000 patients have been enrolled in trials evaluating GP IIb/IIIa inhibitors in non-ST-segment elevation (NSTE) ACS and more than 25 000 have been enrolled in trials evaluating GP IIb/IIIa inhibitors in PCI (Tricoci and Peterson, 2006). For example, in a subgroup of patients who had an ACS event, during 30 days of PCI, GP IIb/IIIa therapy was associated with an even larger reduction in the rate of death or myocardial infarction (MI) of approximately 50% (Braunwald *et al.*, 2002). We used a canine model of left circumflex (LCX) coronary artery CFR, which mimics the clinical condition unstable angina (UA), to evaluate the antithrombotic efficacy of Z4A5 in dogs. This model permits the investigation of CFRs caused by platelet-dependent thrombi formation at the injured, stenotic site of the artery (Wu *et al.*, 2002). We demonstrated that prior to drug or vehicle control administration, endothelial damage to the left anterior descending coronary artery and placement of the coronary constrictor resulted in recurrent CFRs with mean frequencies of 10 to 18 per 60 min observation interval in various groups. Indeed, in all six animals receiving doses of 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} or higher, approximately 100% inhibition of the CFRs was obtained, whereas administration of 30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1} resulted in 39% inhibition. These data form the basis of evidence, which suggests the potential use of the GP IIb/IIIa inhibitor Z4A5 in patients with ACS, particularly UA.

This study was also designed to clarify the antithrombotic effect of Z4A5 on ferric chloride-induced vascular injury and arterial thrombus formation. This model is a modification of a similar injury model in rats (Kurz *et al.*, 1990) and involves oxygen radical formation and specific damage to the endothelium to create a site for platelet adhesion and thrombus. Z4A5 was effective in the ferric chloride model at doses (30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1} to 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}, i.v.) similar to those that were effective in the LCX coronary artery CFR model, reduced thrombus mass and delayed or prevented occlusive thrombus formation in a dose-

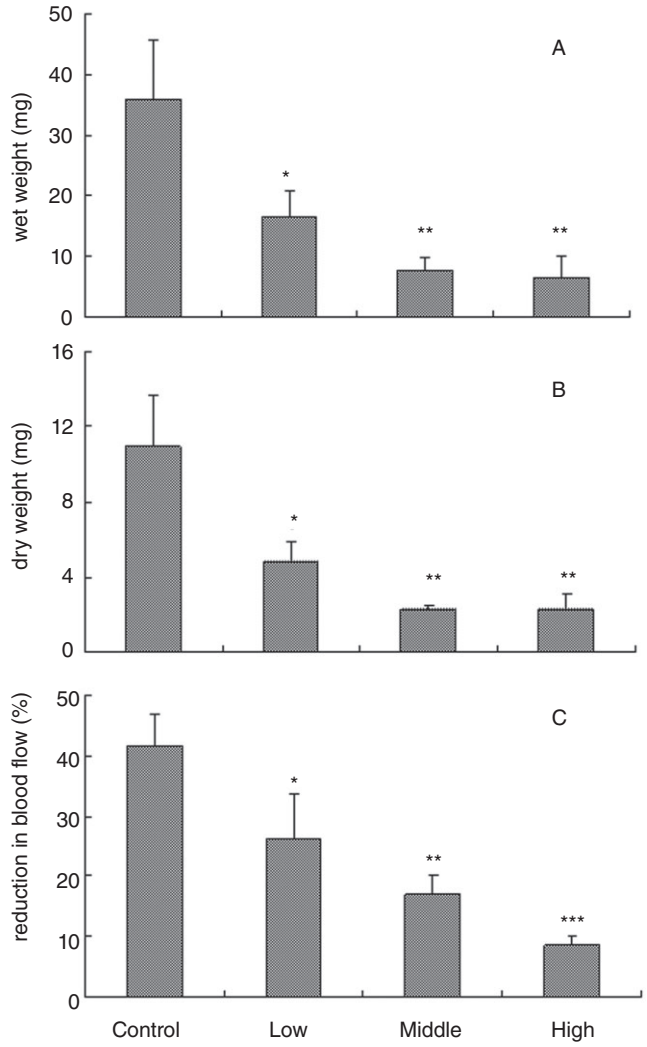

Figure 6

Effect of doses of Z4A5 and control on femoral artery thrombosis. Z4A5 was administered i.v. as a loading bolus followed by a continuous infusion for 60·min at dose of 30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}, 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} or 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}. Values are the means ± SEM, n = 6 for each group: (A) wet thrombus weight, (B) dry thrombus weight, (C) reduction of blood flow. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.

dependent manner. Our results appear to be supported by a previous findings indicating that, in a similar guinea pig model of ferric chloride-induced carotid artery thrombosis, a selective platelet glycoprotein IIb/IIIa receptor antagonist, RWJ-53308, exhibited a dose-dependent increase in mean time to occlusion when administered p.o. (Damiano *et al.*, 2001). These findings indicate significant antithrombotic potential for Z4A5 in the treatment of coronary artery ischaemic syndromes and arterial thrombus formation.

By blocking the final pathway of platelet aggregation, in which platelets bridge with fibrinogen, glycoprotein IIb/IIIa inhibitors can disaggregate platelets and thereby potentially reduce thrombotic complications as well as increase bleeding risk in patients undergoing PCI (Danchin and Aïssaoui,

Table 2

Effects of Z4A5 and control solution on PT and APTT in dogs

	Treatment	0 min	5 min	15 min	30 min	60 min	75 min	90 min	120 min	n	P
PT (s)	Control	7.85 ± 0.16	7.83 ± 0.20	7.63 ± 0.19	7.45 ± 0.13	7.30 ± 0.30	7.50 ± 0.26	7.95 ± 0.42	7.63 ± 0.27	6	ns
	30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}	7.45 ± 0.30	7.62 ± 0.39	7.82 ± 0.63	7.78 ± 0.62	7.63 ± 0.52	7.87 ± 0.46	7.73 ± 0.45	7.67 ± 0.53	6	ns
	30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}	7.63 ± 0.32	7.72 ± 0.30	7.82 ± 0.38	7.78 ± 0.38	7.60 ± 0.33	8.18 ± 0.46	8.02 ± 0.53	7.93 ± 0.44	6	ns
	300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}	7.48 ± 0.38	7.73 ± 0.23	7.97 ± 0.29	7.92 ± 0.30	7.82 ± 0.06	8.22 ± 0.30	7.83 ± 0.20	7.52 ± 0.11	6	ns
APTT (s)	Control	16.08 ± 1.70	15.11 ± 1.57	14.57 ± 1.55	14.55 ± 1.11	12.83 ± 0.80	13.28 ± 0.76	13.77 ± 0.68	13.45 ± 0.38	6	ns
	30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}	12.10 ± 1.26	11.52 ± 0.67	11.92 ± 0.45	11.42 ± 0.51	11.14 ± 0.33	11.20 ± 0.54	10.88 ± 0.38	11.78 ± 0.89	6	ns
	30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}	15.98 ± 1.30	13.78 ± 0.88	14.18 ± 1.12	13.66 ± 0.75	14.76 ± 1.62	14.76 ± 1.30	13.98 ± 1.16	15.42 ± 0.99	6	ns
	300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}	14.48 ± 1.14	13.18 ± 1.69	12.55 ± 1.93	13.08 ± 1.50	12.28 ± 1.03	12.92 ± 1.17	12.45 ± 1.47	13.53 ± 1.69	6	ns

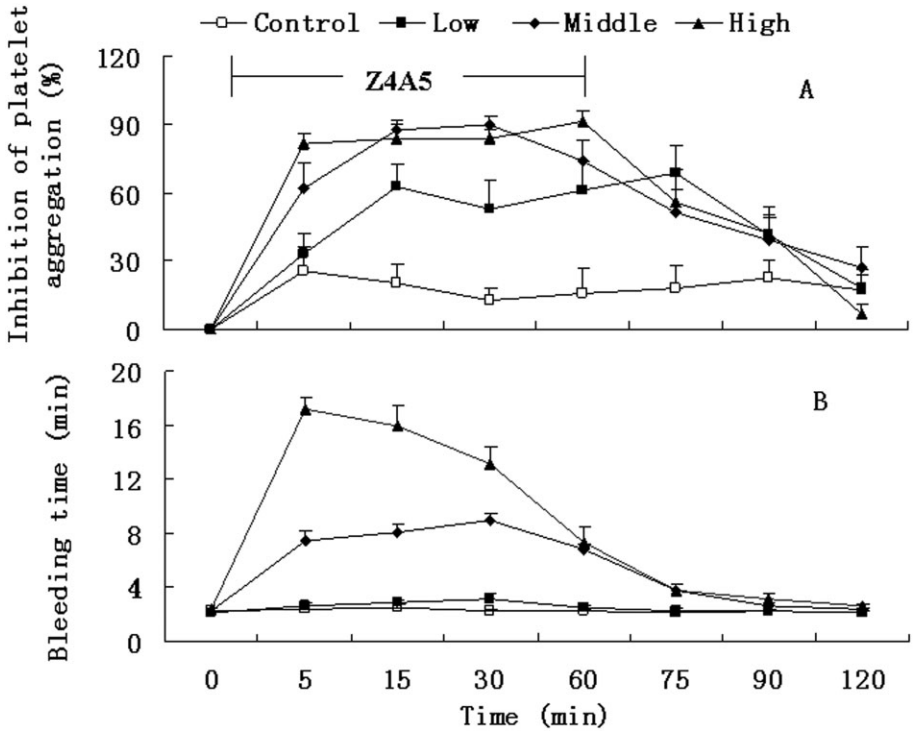

Figure 7

Effects of Z4A5 on (A) ADP-induced canine platelet aggregation *ex vivo* and (B) tongue bleeding time. Z4A5 was administered i.v. as a loading bolus followed by a continuous infusion for 60 min at doses of 30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}, 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} or 300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}. Data are the means ± SEM, n = 6 for each group.

2010). Indeed, bleeding complications are common features of GPIs. Meta-analysis of the use of GP IIb/IIIa inhibitors in ACS suggests that their use leads to a significant increase in major bleeding (2.4% vs. 1.4%, $P < 0.0001$) (Harding *et al.*, 2002). Because of the associated bleeding risk, these agents are only administered within the acute/hospital setting and are not used in the long-term care of patients with atherothrombotic disease (Jennings, 2009). Administration of the lowest Z4A5 dose (30 μg·kg^{-1} + 1 μg·kg^{-1}·min^{-1}) to the dogs in this study revealed that when 50–60% of the platelet aggregation was inhibited, a 39% reduction in CFRs was observed with no prolongation of bleeding time. When an approximate 100% reduction in CFRs was observed after administration of 30 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1} Z4A5, the bleeding time increased by less than threefold. At this dose, more than 80% of platelet aggregation was inhibited, with no dramatic changes in haemodynamic and coagulation parameters. Thus, while the doses required for antithrombotic effects are associated with haemostasis, this risk can be reduced when patients receive appropriate drug dosing (Barrett *et al.*, 2008). In contrast, with an effective dose (95% thrombotic weight reduction and 90% platelet aggregation inhibition) of GPI 7E3 in canines, a greater than eightfold increase in bleeding time was observed (Makkar *et al.*, 1997). Our results suggest that the therapeutic window of Z4A5 may be broader, and fewer bleeding problems may be anticipated when Z4A5 is used as an antithrombotic agent.

The net benefit of GP IIb/IIIa inhibitors is determined by the balance between their therapeutic benefits and risks (Alexander *et al.*, 2006). Some studies have recently shown that the apparent increased mortality due to GP IIb/IIIa antagonists may result from a direct toxic action unrelated to a prothrombotic effect (Chew *et al.*, 2001). A number of hypotheses have suggested that this toxicity may result from interactions with other RGD binding sites, and thus compounds blocking GP IIb/IIIa via another binding site may have an improved toxicity profile (Damiano *et al.*, 2001). Z4A5, a small peptide molecule, was designed based on the spatial structure and character of the RGD motif but does not contain the RGD amino acid sequence; thus, adverse side effects may be minimized with the use of Z4A5. Our previous study demonstrated that the anti-platelet activity of Z4A5 may be due to the block of fibrinogen binding to the GP IIb/IIIa receptor and that Z4A5 did not affect the similar integrin $\alpha_v\beta_3$ (Li *et al.*, 2012). Moreover, this linear structure gives Z4A5 a rapid-on and rapid-off pharmacological profile. In our previous study, the platelet inhibitory action of Z4A5 was apparent immediately, 5 min, after administration and dissipated rapidly upon discontinuing the drug for 15 min (Jing *et al.*, 2011). Meanwhile, a similar phenomenon was observed in the present study; even at the highest i.v. dose of Z4A5 (300 μg·kg^{-1} + 5 μg·kg^{-1}·min^{-1}), platelet aggregation and bleeding time also reverted to baseline 60 min and 15 min after the termination of the infusion respectively. This readily reversible effect of Z4A5 minimizes the likelihood of haemorrhagic problems during the post-treatment period. There appears to be a safe margin for elevation in dose, prolongation of treatment duration or enhanced effect in certain indi-

vidual animals before adverse side effects approached a significant increase over baseline (Lynch *et al*., 1995). Thus, as a consequence of its small molecule peptide structure, Z4A5 may have a greater benefit to risk ratio.

In conclusion, these findings indicate that Z4A5 is an effective antithrombotic agent for coronary and femoral artery thrombosis and could be used as an alternative drug for the treatment of coronary artery ischaemic syndromes. Given its lack of effect on haemodynamic and coagulation parameters and the marginal increase in bleeding time, Z4A5 should be investigated further, to determine its potential as an antithrombotic compound, in the long-term care of animals with acute arterial thrombotic syndromes.

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Conflicts of interest

None.

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