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Research Paper

Anticoagulant, anti-aggregation and antithrombotic effects of a novel hexapeptide

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Abstract

Objectives Hexapeptide is a novel synthetic oligopeptide with a structure similar to that of eptifibatide. This study was designed to investigate the anticoagulant, anti-aggregation, disaggregation and anti-thrombogenesis effects of hexapeptide.

Methods The effects of antiplatelet aggregation induced by adenosine diphosphate (ADP), arachidonic acid (AA) and thrombin, and the effect of disaggregation of platelet aggregation induced by ADP were determined. The anticoagulation indexes were determined by different kits.

Key findings Hexapeptide 1×10^{-5}–1×10^{-4} M could significantly prolong rabbit blood clotting time, thrombin time, prothrombin time and activated partial thromboplastin enzyme time, and reduce the length, wet weight, dry weight and the index of thrombus in a concentration-dependent manner. Hexapeptide 1×10^{-4} M decreased platelet adhesion rate by 40.2%. The platelet aggregation inhibition of hexapeptide in dogs and humans was more obvious than in rabbits and rats. The aggregation inhibition rate of 1×10^{-5} M hexapeptide in dogs, rabbits, rats and humans induced by ADP was 93.9 ± 1.3%, 66.2 ± 1.4%, 76.1 ± 3.2% and 99.8 ± 0.2%, respectively; the 50% inhibitory concentration (IC50) of hexapeptide was 7.24×10^{-8}, 3.24×10^{-6}, 6.61×10^{-6} and 8.91×10^{-8} M, respectively. For the aggregation inhibition rate of hexapeptide in dogs, rabbits and humans induced by AA, the IC50 was 1.29×10^{-9}, 1.32×10^{-6} and 9.33×10^{-8} M, respectively; the IC50 of aggregation inhibition rates induced by thrombin was 2.88×10^{-6}, $>1 \times 10^{-5}$ and 4.17×10^{-6} M, respectively. The disaggregation rate of 1×10^{-4} M hexapeptide in dog induced by ADP was 68.8 ± 7.4%.

Conclusions Hexapeptide has anticoagulant, antiplatelet aggregation, disaggregation and antithrombotic effects *in vitro*.

Keywords adhesion rate; disaggregation; hexapeptide; platelet aggregation; thrombus

Introduction

Platelet aggregation inhibitors can decrease the formation of, or the chemical signals promoting, platelet aggregation. The final step in this response depends on the platelet membrane glycoprotein receptors, which can bind adhesive proteins such as fibrinogen and von Willebrand factor. GPIIb/IIIa is the most important of these receptors and ultimately regulates platelet aggregation and thrombus formation.[1] There are a lot of reports and applications of the significant thrombolytic effects of GPIIb/IIIa receptor antagonists, including abciximab, eptifibatide and tirofiban.[2–5] Thus, platelet activating agents, such as ADP, thromboxane A_2 and thrombin, promote the conformational change necessary for the GPIIb/IIIa receptor to bind ligands, particularly fibrinogen. Fibrinogen simultaneously binds to GPIIb/IIIa receptors on two separate platelets, resulting in platelet cross-linking and aggregation. Platelet aggregation inhibitors are beneficial in the prevention and treatment of occlusive cardiovascular diseases, the maintenance of vascular grafts and arterial patency.[6]

Wang *et al*.[7] synthesized and reported a clot-targeted microbubble that with low-frequency ultrasound showed a thrombolytic effect in an acute arterial occlusion model in rabbits by blocking the common carotid artery with an autogenous clot. The targeted microbubble was based on a specific hexapeptide, whose structure has been reported by Wang *et al*.[8] The specific recognition of this hexapeptide has been confirmed in a canine model in which the clot-targeted microbubble significantly enhanced the ultrasound imaging

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of acute thrombosis in the femoral artery.[9] The targeted microbubbles with low-frequency ultrasound showed significant arterial thrombolysis in the acute arterial occlusion model in rabbits.[7] The hexapeptide, consisting of six amino acids, is similar to eptifibatide, an oligopeptide composed of seven amino acids.[10] Eptifibatide has been used widely in antiplatelet aggregation and antithrombosis in the clinical setting. Our study was to investigate the effects of hexapeptide on anticoagulant, anti-aggregation, disaggregation and antithrombosis *in vitro*, and the hexapeptide is hoped to be a more widely used drug in clinics, including its effect of arterial thrombolysis. Also, the research into the disaggregation effect of the hexapeptide on platelets will explain the arterial thrombolysis mechanism of clot-targeted microbubble to a certain extent.

Materials and Methods

Animals

Rabbits (2.0–2.5 kg), Sprague–Dawley rats (180–220 g) and dogs (10–15 kg) were purchased from Animal Center of Xi'an Jiaotong University College of Medicine. The animals were housed in groups, maintained at 21–24°C on a 12-h light–dark cycle, with free access to water and standard food. The experimental protocols for using the animals had been reviewed and approved by the animal ethics committee at Xi'an Jiaotong University College of Medicine.

Reagents

Hexapeptide was from Dr Bing Wang, Department of Pathology, Xi'an Jiaotong University College of Medicine, China. The purity of the hexapeptide determined by HPLC-MS was 97.06%. Eptifibatide was purchased from Hangzhou Pharmaceutical Co. Ltd (Hangzhou, China). Both hexapeptide and eptifibatide were dissolved in 1% Na_2CO_3 with a double molar amount of each drug under ultrasound, respectively, and then were diluted to the desired concentrations with normal saline. Clopidogrel hydrogen sulfate was purchased from Jinan Lekangxin Co. Ltd (Jinan, China). Dipyridamole injection was from Yabao Pharmaceutical Group Co. Ltd (Yuncheng, China). Aspirin, ADP-Na_2 and arachidonic acid were purchased from Sigma (Santa Clara, CA, USA). Adrenaline hydrochloride injection was provided by Tianjin Jinyao Amino Acid Co. Ltd (Tianjin, China). Thrombin lyophilized powder was from Changchun Guoao Pharmaceutical Group Co. Ltd (Changchun, China). Pentobarbital sodium came from Beijing Solarbio Science & Technology Co. Ltd (Beijing, China). The urea dilute solution: 10 g urea, 0.1 ml formalin, 0.5 g sodium citrate and distilled water were added to 100 ml, dissolved, filtered and preserved at 4°C. Other reagents used in this study were all analytical grade.

Preparation of blood

Rabbits, rats or dogs were anaesthetized by intraperitoneal injection of pentobarbital sodium, and the blood was collected through an arterial cannula. The anticoagulant blood was obtained by adding 3.8% sodium citrate in whole blood. The human anticoagulant blood was kindly provided by Xi'an Blood Center, China.

Determination of coagulation

Tests were grouped: (group 1) control (saline); (groups 2–4) 10^{-6}, 10^{-5} and 10^{-4} M hexapeptide, respectively; (groups 5–7) 10^{-6}, 10^{-5} and 10^{-4} M eptifibatide, respectively. To determine coagulation time (CT), 0.1 ml drug solution and 0.4 ml rabbit blood without anticoagulant were added into EP tubes. The time that the blood did not flow (i.e. CT), was recorded.

Anticoagulant blood was added into EP tubes for centrifugation and the plasma was obtained. The thrombin time (TT), prothrombin time (PT), plasma recalcification time (PRT) and activated partial thromboplastin time (APTT) were determined, respectively, according to the instructions with the kits.

Platelet adhesion

The groups were the same as for the above experiment. To a test tube was added 20 μl drug solution, 20 μl rabbit blood without anticoagulant and 0.38 ml urea dilution. After mixing, a drop was placed onto the blood cell count board and the number of platelets was taken as the platelet count before adhesion. After the mixture was poured into a glass ball, the glass ball was placed into an in-vitro thrombosis platelet adhesion dual-purpose instrument (BJ-9100; Beijing Kejian Sensing Technology Company, Beijing, China). After the glass ball was revolved for 15 min (4 rev/min), the number of platelets was counted as the platelet count after adhesion. Platelet adhesion rate was calculated using the following formula:[11]

$$\text{Platelet adhesion rate}\,(\%) = (\text{Platelet count before adhesion} - \text{Platelet count after adhesion}) \times 100\%/\text{Platelet count before adhesion} \quad (1)$$

Thrombogenesis

The groups were the same as for the above experiment. Drug solution 0.2 ml and rabbit blood 0.8 ml without anticoagulant were poured into a silicone tube (0.5 mm diameter), and the tube was placed in a thrombosis platelet adhesion dual-purpose instrument *in vitro*, and rotated for 15 min. After that, the thrombus formed in the tube was moved onto a piece of filter paper. The length (L, mm) and wet weight (W, mg) of thrombus were measured. The dry weight of thrombus was obtained after the wet thrombus had been baked in an oven at 64°C for 20 min. Thrombus index (Q) was calculated as the following formula.[12]

$$Q = L/2 + W^{1/2} \quad (2)$$

Preparation of platelets

Anticoagulant blood samples of dog, rabbit, rat or human for the measurement of platelet aggregation and disaggregation were centrifuged at 1000 rev/min for 10 min. The supernatant, platelet-rich plasma (PRP), was removed. The remaining blood underwent a second centrifugation step at a speed of 3000 rev/min for 10 min to gain platelet-poor plasma (PPP). PRP was diluted with autologous PPP to adjust its platelet count to 250/nl.

Platelet aggregation inhibition

According to the literature method,[3] platelet aggregation was determined by light transmission in a four-channel platelet aggregation analyser (LBY-NJ4; Beijing Precil Instrument Co. Ltd, Beijing, China). The transmittancy was adjusted with PRP to 0 and with PPP to 100% before each test. Firstly, in a sample cup, 300 μl PRP was added, then, saline, hexapeptide (final concentration: 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8} and 1×10^{-9} M), eptifibatide (final concentration, 1×10^{-6} M), clopidogrel (final concentration, 3×10^{-5} M), dipyridamole (final concentration, 2×10^{-6} M) or aspirin (dissolved in 1% Na_2CO_3 and adjusted pH to 7.0 with HCl; final concentration: 2.8×10^{-4} M)[13] was added, too. Secondly, the sample cup was cultured at 37°C for 20 min. Thirdly, platelets in 300 μl PRP were stimulated by the addition of ADP, AA (dissolved to 10% solution with absolute alcohol and diluted to the required concentration with 1% Na_2CO_3 before using)[14] or thrombin and were stirred by magneton. Adrenaline 2 μM was added into the platelet aggregation or disaggregation system induced by ADP to enhance the induction effects of ADP in dogs, rabbits, rats and humans. As soon as the magneton was added in the sample cup, the platelet aggregation curve within 5 min was recorded. The inhibiting rate of platelet aggregation was calculated as follows:

$$\text{Inhibiting rate of platelet aggregation}\,(\%) = (\text{Aggregation of control} - \text{Aggregation of drugs})/\ \text{Aggregation of control} \times 100\% \quad (3)$$

Platelet disaggregation

PRP and PPP of dogs were used to study platelet disaggregation. The transmittancy was adjusted with PRP to 0 and with PPP to 100% before each test. Platelets in 300 μl PRP were pre-cultured at 37°C for 10 min and were stimulated by ADP while stirring. Saline (control) or drug (final concentration: 1×10^{-4}, 1×10^{-5}, 3×10^{-6}, 1×10^{-6}, 3×10^{-7}, 1×10^{-7} and 1×10^{-8} M) was added into the cups between 30 and 60 s (the aggregation curve reached half of the expected maximal level during the time period) after initiation of aggregation. Disaggregation potency (DP) was calculated as follows:[3]

$$\text{DP}\,(\%) = [1 - (\text{Maximum aggregation of drugs}/\ \text{Maximum aggregation of control})] \times 100\% \quad (4)$$

Statistical analysis

All data were expressed as means ± SEM. SPSS13.0 software and one-way analysis of variance were used to test the differences between groups. $P < 0.05$ was considered statistically significant. Charts were constructed by use of GraphPad Prism 5.0.

Results

Anticoagulation

Table 1 shows the anticoagulation effect of hexapeptide on rabbit blood. Hexapeptide (1×10^{-4} M) significantly increased CT, TT, PT, PRT and APTT compared with control ($P = 0.000$, 0.034, 0.020, 0.000 and 0.045, respectively); the CT and PRT of 1×10^{-5} M hexapeptide were significantly longer than those of control ($P = 0.014$ and 0.002, respectively). This suggests that hexapeptide can prolong the CT, TT, PT, PRT and APTT of rabbit blood and that the anticoagulation effect of hexapeptide is similar to that of eptifibatide.

Antiplatelet adhesion

Table 1 shows the antiplatelet adhesion effect of hexapeptide in rabbits. The adhesion rate of 1×10^{-4} M hexapeptide was significant lower than the control ($P = 0.005$) and 1×10^{-5} M hexapeptide had a tendency to decrease adhesion rate ($P = 0.062$), suggesting that hexapeptide can inhibit platelet adhesion in the rabbit.

Antithrombosis

Hexapeptide (1×10^{-4} M) and eptifibatide (1×10^{-4} M) significantly decreased the length, wet weight, dry weight and index of thrombus compared with control ($P = 0.009$, 0.039, 0.043 and 0.005, and $P = 0.001$, 0.030, 0.002 and 0.002, respectively). There wasn't any significant difference in thrombotic indexes between 1×10^{-4} M hexapeptide and 1×10^{-4} M eptifibatide ($P > 0.05$) (Table 2). Hexapeptide 1×10^{-5} M had a tendency to decrease the length, wet weight, dry weight and index of thrombus.

Platelet aggregation inhibition

Dogs

When ADP was used as a platelet aggregation inducer, the platelet aggregation rates of 1×10^{-8} to 1×10^{-5} M

Table 1 The effect of hexapeptide on whole blood clotting time, thrombin time, prothrombin time, plasma recalcification time, activated partial thromboplastin time and plasma adhesion rate in rabbits *in vitro*

Drug	Concentration (mol/l)	CT (min)	TT (s)	PT (s)	PRT (min)	APTT (s)	Adhesion rate (%)
Control	–	3.98 ± 0.28	16.7 ± 2.3	5.5 ± 0.8	5.9 ± 0.6	26.0 ± 1.5	40.3 ± 2.9
Hexapeptide	10^{-4}	7.70 ± 0.46**	22.3 ± 2.1*	10.7 ± 1.9*	8.7 ± 0.5**	30.6 ± 2.6*	24.1 ± 3.4**
	10^{-5}	5.74 ± 0.62*	18.1 ± 2.0	7.6 ± 1.0	8.0 ± 0.5**	27.3 ± 2.8	31.9 ± 2.8
	10^{-6}	4.64 ± 0.50	12.7 ± 1.4	5.9 ± 0.7	7.1 ± 0.6	24.9 ± 2.6	45.4 ± 2.3
Eptifibatide	10^{-4}	7.53 ± 0.35**	27.0 ± 2.2**	15.2 ± 2.2**	7.4 ± 0.7*	29.8 ± 1.7*	25.9 ± 3.6*
	10^{-5}	5.46 ± 0.57*	21.1 ± 2.2	8.5 ± 1.0*	6.9 ± 0.7	25.4 ± 1.7	35.5 ± 1.9
	10^{-6}	4.52 ± 0.53	16.1 ± 2.4	6.8 ± 0.9	6.4 ± 0.7	22.3 ± 1.6	45.2 ± 2.4

APTT, activated partial thromboplastin time; CT, whole blood clotting time; PRT, plasma recalcification time; PT, prothrombin time; TT, thrombin time. Data are means ± SEM, $n = 6$. *$P < 0.05$, **$P < 0.01$ vs control.

Table 2 Antithrombosis effect of hexapeptide in rabbit *in vitro*

Drug	Concentration (mol/l)	Thrombus			
		Length (mm)	Wet weight (mg)	Dry weight (mg)	Index
Control	–	74 ± 5	237 ± 27	70 ± 8	52.4 ± 3.1
Hexapeptide	10^{-4}	50 ± 6**	158 ± 22*	43 ± 9*	37.5 ± 3.1**
	10^{-5}	77 ± 9	231 ± 19	51 ± 9	53.9 ± 5.3
	10^{-6}	86 ± 4	308 ± 25	88 ± 17	60.5 ± 2.4
Eptifibatide	10^{-4}	54 ± 5*	151 ± 24*	40 ± 7*	39.2 ± 2.5**
	10^{-5}	66 ± 11	226 ± 35	63 ± 11	48.1 ± 6.9
	10^{-6}	77 ± 13	233 ± 37	68 ± 13	53.6 ± 7.7

Data are means ± SEM, $n = 6$. *$P < 0.05$, **$P < 0.01$ vs control.

Table 3 Platelet aggregation rates (%) of hexapeptide induced by adenosine diphosphate, arachidonic acid and thrombin in dogs and rabbits *in vitro*

Drug	Concentration (mol/l)	Dog			Rabbit		
		ADP (50 μM)	AA (1.0 mM)	Thrombin (0.4U/ml)	ADP (20 μM)	AA (1.0 mM)	Thrombin (1.0U/ml)
Control	–	54.5 ± 1.1	46.6 ± 3.8	54.4 ± 3.1	57.0 ± 3.8	40.1 ± 2.5	51.7 ± 2.1
Hexapeptide	10^{-5}	3.3 ± 1.3**	0.2 ± 0.5**	19.4 ± 1.7**	29.2 ± 3.4**	25.1 ± 2.2**	41.3 ± 3.2*
	10^{-6}	6.3 ± 1.2**	1.1 ± 0.7**	37.0 ± 3.8*	41.3 ± 3.0**	26.8 ± 2.4**	42.0 ± 3.3*
	10^{-7}	31.2 ± 3.2**	1.4 ± 0.6**	38.6 ± 4.7*	42.7 ± 2.7*	31.6 ± 2.1*	46.2 ± 4.1
	10^{-8}	43.9 ± 3.7*	8.5 ± 0.7**	38.9 ± 5.7	43.7 ± 1.6	32.7 ± 2.5	55.6 ± 3.6
	10^{-9}	47.0 ± 5.6	26.5 ± 3.6**	47.6 ± 4.2	43.7 ± 3.7	35.4 ± 2.3	54.6 ± 4.7
Eptifibatide	10^{-6}	4.2 ± 1.6**	0.4 ± 0.2**	34.6 ± 1.9**	19.3 ± 2.5**	25.3 ± 2.6**	45.8 ± 2.6
Clopidogrel	3×10^{-5}	5.9 ± 0.6**	10.0 ± 1.0**	55.5 ± 2.8	35.7 ± 3.1**	27.2 ± 1.2**	51.6 ± 2.4
Dipyridamole	2×10^{-5}	47.4 ± 5.0	11.7 ± 2.4**	46.8 ± 5.0	45.7 ± 3.9	20.6 ± 3.2**	42.5 ± 1.8**
Aspirin	2.8×10^{-4}	31.9 ± 2.4**	0.6 ± 0.2**	12.9 ± 3.0**	19.3 ± 2.5**	2.1 ± 0.6**	44.4 ± 3.6

AA, arachidonic acid; ADP, adenosine diphosphate. Data are means± SEM, $n = 6$. *$P < 0.05$, **$P < 0.01$ vs control.

hexapeptide varied from 3.3% to 43.9%, which was significant lower than the control (54.5 ± 1.1%, $P = 0.021$, 0.000, 0.000 and 0.000, respectively) (Table 3); There was significant difference in the platelet aggregation rates between different concentrations of hexapeptide ($P < 0.01$), suggesting that the aggregation inhibition is concentration dependent. There was no significant difference in platelet aggregation rates between 1×10^{-6} M hexapeptide and 1×10^{-6} M eptifibatide. Table 3 also shows the platelet aggregation rate induced by AA. The aggregation rate of the control was 46.6 ± 3.8%. The platelet aggregation inhibition rates of 1×10^{-9} to 1×10^{-5} M hexapeptide were 13.7 ± 3.6%, 19.4 ± 0.7%, 42.3 ± 0.6%, 88.5 ± 0.7% and 93.9 ± 1.3%, respectively, which was significant lower than the control ($P < 0.01$). The inhibition effect of hexapeptide on platelet aggregation induced by AA was significant stronger than its effect on the aggregation induced by ADP. When thrombin was used as a platelet aggregation inducer, the platelet aggregation rates (38.6–19.4%) of 1×10^{-7} to 1×10^{-5} M hexapeptide were obviously lower than control (54.4 ± 3.1%) ($P = 0.013$, 0.010 and 0.000, respectively). The inhibition effect of hexapeptide on platelet aggregation induced by ADP or AA was stronger than that of aggregation induced by thrombin. Aspirin 2.8×10^{-4} M significantly inhibited dog platelet aggregation induced by the three inducers ($P < 0.01$).

The concentration–inhibition curves of hexapeptide and eptifibatide on dog platelet aggregation induced by ADP, AA or thrombin were similar. The 50% inhibitory concentration (IC50) values of hexapeptide inhibiting dog platelet aggregation induced by ADP, AA and thrombin were 7.24×10^{-8} M, 1.29×10^{-9} M and 2.88×10^{-6} M, and those of eptifibatide were 5.74×10^{-8} M, 6.76×10^{-10} M and 1.62×10^{-6} M, respectively (Figure 1).

Rabbits

The platelet aggregation rate of the control induced by ADP was 57.0 ± 3.8% (Table 3). Hexapeptide (1×10^{-7} to 1×10^{-5} M) obviously decreased the platelet aggregation rates ($P = 0.011$, 0.009 and 0.000), and the aggregation inhibition rate of 1×10^{-5} M hexapeptide was 66.2 ± 1.4%. The platelet aggregation rate of the control induced by AA was 40.1 ± 2.5% and in the same way, hexapeptide (1×10^{-7} to 1×10^{-5} M) obviously decreased the platelet aggregation rate ($P = 0.028$, 0.003 and 0.001). This showed that the platelet aggregation rate of control induced by thrombin was 51.7 ± 2.1%. The platelet aggregation rates of hexapeptide 1×10^{-6} M and 1×10^{-5} M were significantly lower than that of control ($P = 0.034$ and 0.022, respectively). The IC50 values of hexapeptide inhibiting rabbit platelet aggregation induced by ADP, AA and thrombin were less than 1×10^{-5} M. IC50 values for aggregation induced by ADP, AA and thrombin were 3.24×10^{-6} M, 1.32×10^{-6} M and $>1 \times 10^{-5}$ M, respectively (Figure 4a). The results suggest that hexapeptide can inhibit rabbit platelet aggregation.

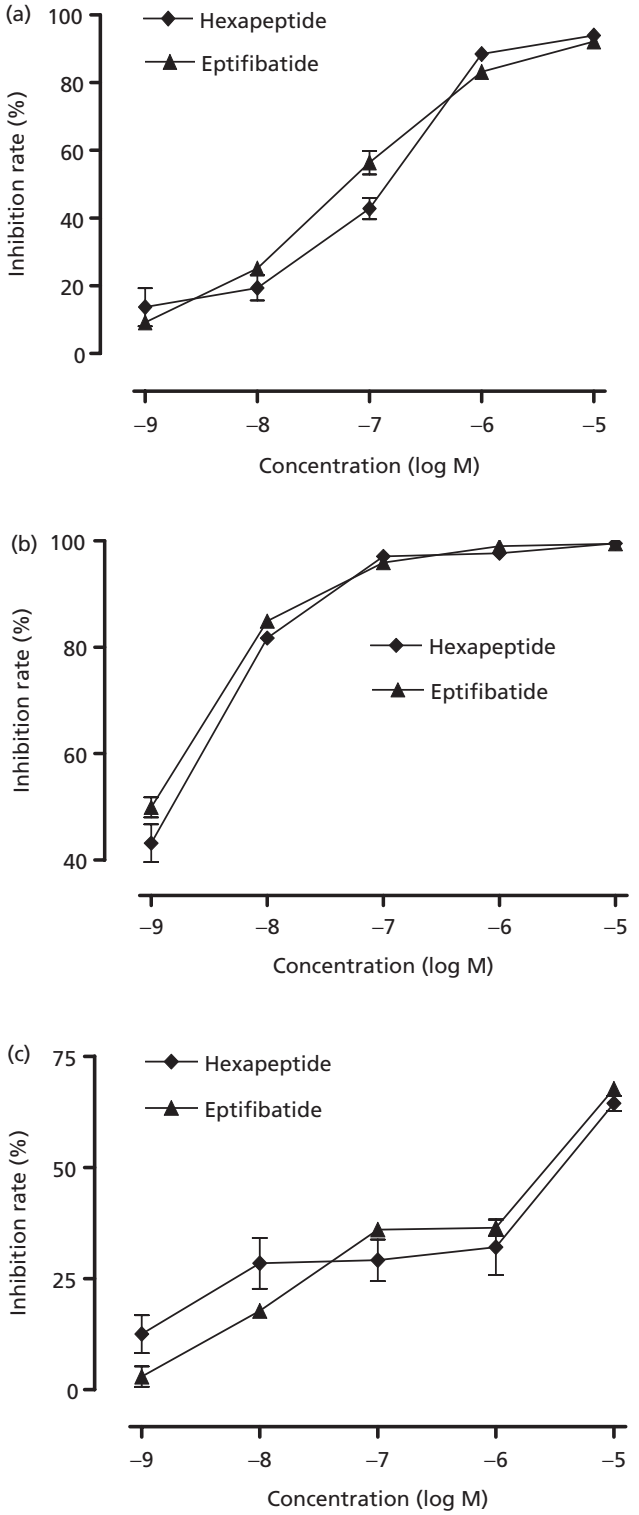

Figure 1 The concentration–inhibition curves of hexapeptide on dog platelet aggregation induced by 50 μM adenosine diphosphate (a), 1.0 mM arachidonic acid (b) and 0.4 U/ml thrombin (c). Data are means ± SEM, $n = 6$. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control.

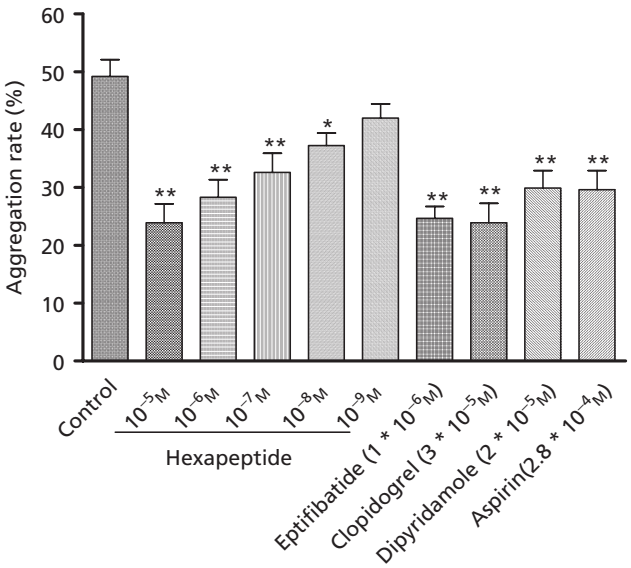

Figure 2 Inhibitory effect of hexapeptide on rat platelet aggregation induced by 50 μM adenosine diphosphate. Data are means ± SEM, $n = 6$. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control.

Rats

Rat platelet aggregation rate of the control induced by ADP was 49.2 ± 2.9%, and the aggregation rates of 1×10^{-8} to 1×10^{-5} M hexapeptide varied from 37.3% to 23.9%, which was significant lower than control ($P = 0.017$, 0.006, 0.001 and 0.000, respectively), and the aggregation inhibition rate of 1×10^{-5} M hexapeptide was 76.1 ± 3.2% (Figure 2). The IC50 of hexapeptide for rat platelet aggregation inhibition was 6.61×10^{-6} M.

Humans

The platelet aggregation rates of 1×10^{-8} to 1×10^{-5} M hexapeptide induced by ADP varied from 0.1% to 38.6%, which was significantly lower than control (47.7 ± 2.2%) ($P = 0.005$, 0.000, 0.000 and 0.000, respectively), and the aggregation inhibition rate of 1×10^{-5} M hexapeptide was 99.8 ± 0.2%. Hexapeptide showed a concentration-dependent inhibitory effect on human platelet aggregation (Figure 3a). The platelet aggregation rates of 1×10^{-9} to 1×10^{-5} M hexapeptide induced by AA were significant lower than control (42.3 ± 1.3%) ($P = 0.001$, 0.000, 0.000, 0.000 and 0.000, respectively) (Figure 3b). When thrombin was used as a platelet aggregation inducer, the platelet aggregation rates of 1×10^{-8} to 1×10^{-5} M (43.2–22.7%) hexapeptide were obviously lower than the control (56.0 ± 1.6%) (all $P = 0.000$) (Figure 3c). The IC50 values of hexapeptide inhibiting human platelet aggregation induced by ADP, AA and thrombin were 8.91×10^{-8} M, 9.33×10^{-8} M and 4.17×10^{-6} M, respectively. The IC50 of eptifibatide inhibiting human platelet aggregation induced by ADP was 1.00×10^{-7} M (Figure 4b).

Disaggregation

When the disaggregation was investigated, drugs were added to the sample cups after induction of dog platelet aggregation

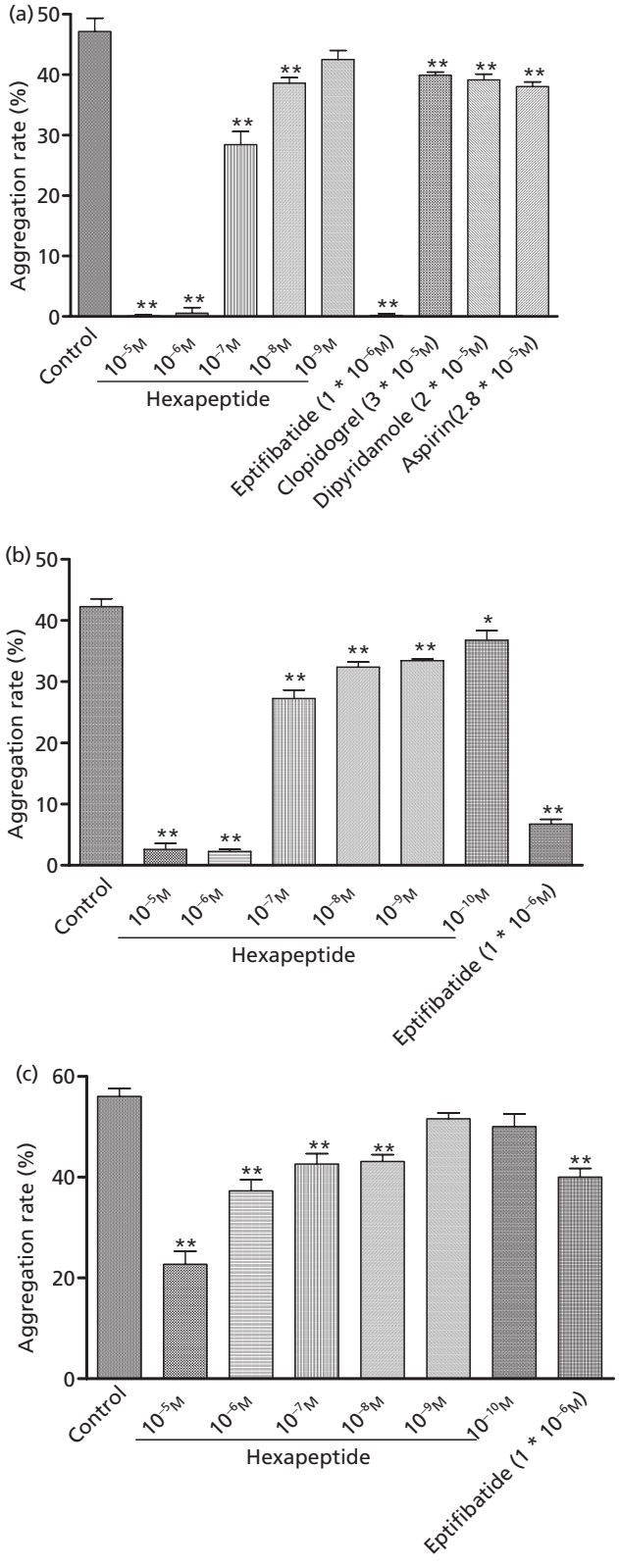

Figure 3 Inhibitory effect of hexapeptide on human platelet aggregation induced by 20 μM adenosine diphosphate (a), 1.0 mM arachidonic acid (b) and 1.0 U/ml thrombin (c). Data are means ± SEM, $n = 6$. *$P < 0.05$ and **$P < 0.01$ vs control.

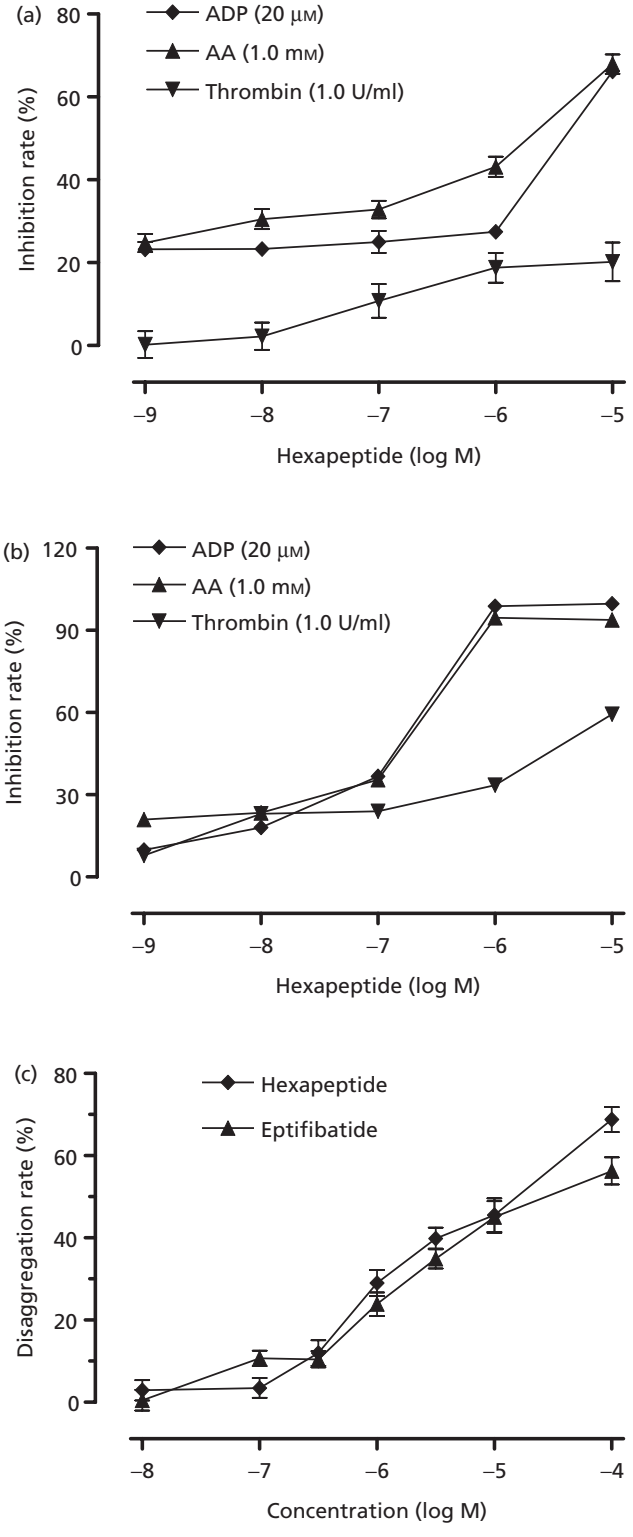

Figure 4 The concentration–inhibition curves of hexapeptide on rabbit platelet aggregation induced by 20 μM adenosine diphosphate (ADP), 1.0 mM arachidonic acid (AA) and 1.0 U/ml thrombin (a). The concentration–inhibition curves of hexapeptide on human platelet aggregation induced by 20 μM ADP, 1.0 mM AA and 1.0 U/ml thrombin (b). Disaggregation effects of hexapeptide and eptifibatide on dog platelets induced by 50 μM ADP (c). Data are means ± SEM, $n = 6$.

by ADP to study thrombolysis. Figure 4c shows that the disaggregation rates increased with the concentrations (final concentration, 1×10^{-8} to 1×10^{-6} M) of hexapeptide (2.9–68.8%) and eptifibatide (0.4–56.3%) increasing, and their disaggregation rates (concentration, 3×10^{-7} to 1×10^{-4} M) resulted in significant higher platelet disaggregation rates compared with the control (all the $P = 0.000$). The disaggregation rates of both hexapeptide and eptifibatide showed no significant difference at the same experimental concentration. The 50% effective concentration (EC50) of platelet disaggregation of hexapeptide and eptifibatide were 1.55×10^{-5} M and 2.95×10^{-5} M, respectively.

Discussion

The occurrence and development of many cardiovascular and cerebrovascular diseases, such as cerebral thrombosis, deep vein thrombosis, pulmonary embolism, myocardial infarction and disseminated intravascular coagulation, are closely related with thrombus formation. For the prevention and treatment of thrombosis, the administration of an anticoagulant drug is essential. Blood platelets play an important role in haemostasis and thrombosis, and the platelet membrane glycoprotein (GP) II b/III a receptor plays a key role in mediating platelet aggregation. Eptifibatide, a cyclic heptapeptide based on a peptide recognition sequence found in snake venom, is one of the specific inhibitors in this class of drugs and has been studied in a broad range of ischaemic coronary conditions. Hexapeptide is similar to eptifibatide in its structure.

Platelet activation can be induced by inducers such as ADP, TXA_2, thrombin, collagen, 5-hydroxytryptamine and prostaglandins. The shape of activated platelets turns from discoid to spherical, then the GPIIb/IIIa receptors on the platelet surface involved in platelet aggregation, and the platelet adhesion increase.[15] GPIIb/IIIa receptor antagonists, such as abciximab, eptifibatide and tirofiban, inhibit platelet aggregation via the final common aggregated pathway.[3] Among them, eptifibatide was chosen as positive control. Otherwise, AA, not TXA_2, was used as a platelet inducer for the corresponding pathway because TXA_2 decomposes in water. Free AA converts to the major metabolite TXA_2 under the action of cyclooxygenase.[16,17] Clopidogrel, aspirin and dipyridamole were used as positive control drugs. Clopidogrel, an ADP receptor antagonist, can irreversibly inhibit phase I and II of platelet aggregation induced by ADP. Aspirin, a cyclooxygenase inhibitor, can inhibit platelet aggregation by inhibiting the activation of cyclooxygenase and blocking the TXA_2 receptor on the platelet membrane. Phosphodiesterase inhibitors, such as dipyridamole, block platelet aggregation by inhibiting the activity of phosphodiesterase, activating adenylyl cyclase and promoting the formation of prostaglandin I_2 by vascular endothelial cells. Different kinds of antiplatelet aggregation drugs as positive controls were used in this study, which will lay the foundation for further research into the hexapeptide combining with other drugs in anticoagulation.

The concentration of ADP, AA or thrombin was based on existing literature. The final concentrations of ADP were 2 μM,[18] 10 μM[19] and 20 μM.[20] Adrenaline 1 μM[21] could increase the induction effect of ADP; the final concentrations of AA were 1 mM,[19] 0.5 mM and 5 mM.[22] The final concentrations of thrombin were 0.1 U/ml,[22] 0.6 U/ml[23] and 1.0 U/ml.[24] In the study, platelet aggregation induced by ADP (enhanced by 2 μM adrenaline) in dog, rabbit, rat and human plasma, the concentrations of ADP were 50 μM, 20 μM, 50 μM and 10 μM, respectively. The optimum concentrations of inducers were closely related to solvent system, platelet source, pre-culture time and magneton size, and so on.

This study showed that the hexapeptide inhibited the platelet aggregation obviously in dog, rabbit and rat. The IC50 value of the hexapeptide inhibiting platelet aggregation induced by different inducers in dog was the most similar to human. The strength order of hexapeptide inhibiting platelet aggregation induced by different inducers was AA > ADP > thrombin in human or dog. The results suggest that the dog is the best animal in the study of platelet aggregation. Scarborough *et al*.[25] reported that the IC50 values of abciximab, tirofiban and eptifibatide inhibiting human platelet aggregation induced by ADP were 5 nM, 15 nM and 120 nM, respectively. In this study, the IC50 for inhibiting platelet aggregation of hexapeptide (8.91×10^{-8} M) in humans induced by ADP was smaller than that of eptifibatide (1.00×10^{-7} M).

After activation by an inducer, platelets may release some endogenous substances that enhance the platelet aggregation. This study showed that the platelet aggregation induced by a specific inducer was inhibited by not only the specific antagonists, but also by other antagonists involved in different pathways. So, when AA induced platelet aggregation, not only aspirin but also clopidogrel and dipyridamole showed anti-aggregation effects.

The concentration–disaggregation curve of hexapeptide was similar to that of eptifibatide (Figure 4c). The EC50 of hexapeptide on dog platelets induced by ADP was 1.55×10^{-5} M, which is 214 times its anti-aggregation IC50 (7.24×10^{-8} M), suggesting that the anti-aggregation effect of hexapeptide on dog platelets induced by ADP exceeded its disaggregation effect. The study of anti-adhesion and anti-thrombosis in rabbits showed that hexapeptide could reduce the adhesion rate and the formation of thrombus.

Platelet aggregation is an important part of the process of blood coagulation; inhibiting platelet aggregation can affect blood coagulation inevitably. CT, PRT, APTT, TT and PT are important indicators of blood coagulation. This study showed that hexapeptide could prolong CT, PRT, APTT, TT and PT, suggesting that hexapeptide has endogenous and extrinsic anticoagulant effects.

As a similar kind of drug to the hexapeptide, eptifibatide has been applied widely in the clinical setting in acute coronary syndrome[5,26] and percutaneous coronary intervention.[27] Hexapeptide targeted microbubbles could enhance the echo of acute thrombosis in the femoral vein in dogs[28] and intra-arterial or intravenous administration of hexapeptide targeted microbubbles could produce a significant thrombolytic effect in acute middle cerebral artery embolism in rabbits,[7] suggesting that the hexapeptide is a potential thrombolytic drug. In the study hexapeptide decreased the length, wet weight, dry weight and index of thrombus, which was similar to the effects of eptifibatide. The hexapeptide showed significant effects of antiplatelet aggregation and disaggregation as did eptifibatide. So, we suggest that this hexapeptide is a GPIIb/

IIIa receptor antagonist too. The mechanisms of its antiplatelet aggregation, disaggregation and thrombolysis are being investigated by us now.

Conclusions

Hexapeptide has the excellent effects of anticoagulation, anti-platelet aggregation, disaggregation and antithrombosis *in vitro*. It is a potential anticoagulant and antithrombotic drug similar to eptifibatide.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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