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Research Paper

Anticoagulant and antithrombotic activity of a new peptide pENW (pGlu-Asn-Trp)

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Abstract

Objectives The aim was to test a newly discovered oligopeptide, pENW (pGlu-Asn-Trp), for its anticoagulant and antithrombotic activity *in vivo*, and try to investigate its underlying mechanisms.

Methods We measured coagulation time by the glass slide method and bleeding time by cutting of mice tails. The thrombosis models employed here included an arterio-venous shunt model and inferior vena ligation model. An ELISA (enzyme-linked immunosorbent assay) was used to analyse t-PA/PAI (tissue-type plasminogen activator/plasminogen activator inhibitor) in the blood drawn from the rats with thrombosis. The ultrastructural changes of the endothelium in the vessels developing thrombosis were observed under a transmission electron microscope.

Key findings We found that pENW-treated mice exhibited a prolonged coagulation time in a dose-dependent manner, but not an extended haemorrhage time. On the other hand, pENW significantly inhibited thrombus formation in both arterio-venous shunt models and inferior vena ligation models. Plasma t-PA/PAI was significantly higher as measured by ELISA. Transmission electron microscope photos of pENW-treated groups also displayed a better condition than model controls, with less erythrocytes in the vascular lumens. In addition, pENW concentration-dependently inhibited aggregation of platelets induced by ADP (adenosine 5′-diphosphate sodium salt) in rabbit platelet-rich plasma.

Conclusions These findings support the suggestion that pENW possesses antithrombotic activity and could be a promising drug in the prevention and treatment of unwanted clot formation.

Keywords anticoagulant; antiplatelet; antithrombotic; new peptide; t-PA/PAI

Introduction

Thrombosis can occur in the arterial or the venous circulation and has a major medical impact. Thrombotic disease, including coronary thrombosis, pulmonary embolism, intracranial embolism and thrombosis, deep venous thrombosis etc, threatens human life to a large extent. Acute arterial thrombosis is the proximal cause of most cases of myocardial infarction (heart attack) and about 80% of strokes, collectively the most common cause of death in the developed world. Venous thromboembolism is the third leading cause of cardiovascular-associated death.

In broad terms, arterial thrombosis is treated with drugs that target platelets and venous thrombosis is treated with drugs that target proteins of the coagulation cascade.[1] Antiplatelet drugs, vitamin K antagonists, unfractionated heparin and low-molecular-weight heparins are the anticoagulants most commonly used for preventing and treating thromboembolic disorders. However, their efficacy is not optimal and their use is associated with a number of disadvantages – notably in terms of safety and convenience of use.[2,3] For example, antiplatelet agents (such as aspirin), recently developed drugs (like the thienopyridines ticlopidine and clopidogrel) and antagonists (abciximab, eptifibatide) of glycoprotein IIb/IIIa[4] that inhibit platelet aggregation and thromboxane A_2 synthetase, have side effects including gastrointestinal symptoms and haemorrhage.[5] The anticoagulation response to unfractionated heparin varies, and its use requires routine laboratory monitoring. Moreover, unfractionated heparin and low-molecular-weight heparins are associated, in a small but

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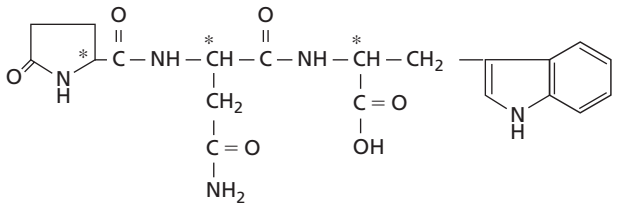

Figure 1 The chemical structure of pENW (pGlu-Asn-Trp). There are three chiral centres (*), all of which are left-handed.

significant proportion of patients, with the occurrence of heparin-induced thrombocytopenia, a severe and life-threatening event.[1] Vitamin K antagonists have numerous limitations, including drug–drug interactions, the need for routine laboratory monitoring, and a high variability of response between patients.[3] As a result, development is needed of new antithrombotic drugs with a better benefit-to-risk ratio and that are more convenient to use in practice.

pENW (pGlu-Asn-Trp) used to be derived from *Agkistrodon acutus* Guenther venom, but now can be synthesized chemically in a simple procedure; its chemical structure is shown in Figure 1. It attracted our attention by being stable. Many studies have demonstrated that snake venom contains a variety of proteins and polypeptides that affect thrombosis and haemostasis.[6–13] For example, *Agkistrodon acutus* Guenther venom contains plenty of effective substances that have act on thrombosis and haemostasis, including fibrinolytic enzymes, thrombin-like enzymes, antagonists of platelet aggregation, coagulation factor IX/X-binding proteins etc.[14–16] So many ingredients together can cause severe bleeding, making the venom a poison. However, of all the ingredients that have been isolated and studied, there has been no report of one that has therapeutic activity as well as a simple structure. As we know, high-molecular-weight proteins or polypeptides tend to stimulate an immune reaction, which creates a problem in some people and limits their usage. As a result, looking for an effective and safe new drug is a priority.

In the study, we investigated the antithrombotic activity of pENW, a newly discovered peptide, whose molecular weight is just 429. We proved its activity in three aspects – its ability to inhibit the process of coagulation and fibrin formation, its effect on the plasma fibrinolysis system and its effect on vascular endothelium and platelets. It is structurally simple, and its stable nature and low molecular weight make it more outstanding than traditional antithrombotic drugs, with potential to be developed for the prevention and treatment of both venous and arterial thrombosis, and commendable for further research.

Materials and Methods

Animals and materials

Sprague-Dawley rats, 250–280 g, and ICR mice, 18–22 g, were purchased from the Comparative Medicine Center, Yangzhou University. They were kept in a temperature-controlled environment (22 ± 2°C), with 55 ± 5% relative humidity and a 12-h light–dark cycle, and fed with standard chow, for at least 1 week before any manipulations. All protocols were approved by the University Ethics Committee on Animal Research and conformed to National Institutes of Health Guideline for Care and Use of Laboratory Animals, in accordance with international accepted principles.

pENW was synthesized by the School of Life Science and Technology, China Pharmaceutical University. Aspirin (for in-vivo study), urokinase and low-molecular-weight heparin calcium (LMWHCa) were produced by Nanjing Baijingyu Pharmaceutical Co. Ltd, Livzon Pharmaceutical Group Inc. and Tianjin Chasesun Pharmaceutical Co. Ltd, China, respectively. The drugs, except aspirin (dissolved in 0.5% sodium carboxymethyl cellulose and administered intragastrically), were dissolved in physiological saline immediately before use to obtain the required concentrations, and were given by intravenous injection. Aspirin (for in-vitro study) was provided by Nanjing pharmaceutical factory. ADP was purchased from Sigma-Aldrich (Shanghai) Trading Co. Ltd. tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI) ELISA kit were purchased from ADL Co.

Coagulation and haemorrhage assay

The coagulation time was detected with the slide method.[17,18] Fifty healthy mice, 25 males and 25 females, were selected and randomly divided into five groups containing 10 mice in each group. There were five males and five females in each group. Physiological saline (20 ml/kg) or pENW (2, 4, 8 mg/20 ml per kg) was administered through tail veins only once, while aspirin (20 mg/20 ml per kg) was administered intragastrically once daily for seven days. Five minutes after the venous injection or 1 h after the last administration of aspirin, a glass capillary was inserted into the mouse medial canthus. A drop of blood was placed on a slide surface. With needle stirring from the edge to the centre of the drop at an interval of 10 s, the coagulation time was measured from the moment the blood was placed on the slide until a fibrin silk was stirred.

The haemorrhage time was determined using a modified tail cutting method.[17,18] Fifty healthy mice, 25 males and 25 females, were selected and randomly divided into five groups containing 10 mice in each group. There were five males and five females in each group. Physiological saline (20 ml/kg) or pENW (2, 4, 8 mg/20 ml per kg) was administered through tail veins, as well as low-molecular-weight heparin calcium (800 IU/20 ml per kg). Five minutes after the administration, the mouse tail was transected 0.5 cm from the tip using a disposable surgical blade. The haemorrhage time was measured from the moment of transection until bleeding completely stopped; the blood was absorbed with filter paper at 20-s intervals during bleeding.

Antithrombotic activity analysis

Arterio-venous shunt model in rats

The method of Umetsu and Sanai[19] was employed with slight modifications.[13,20] Male Sprague-Dawley rats, anaesthetized with ethylcarbamate (1 g/kg), were fixed in the supine position. pENW (1, 2 and 4 mg/5 ml per kg), urokinase (10 000 U/5 ml per kg) or physiological saline (5 ml per kg) was injected through tail veins only once, and aspirin (10 mg/5 ml per kg)

was administered intragastrically once daily for seven days. Five minutes after the venous injection or 1 h after the last administration of aspirin, a cervical incision was made in the midline to expose the left carotid artery and right jugular vein. A 12-cm long polyethylene tube with a 6-cm long silk thread fixed in its lumen was filled with physiological saline. One end of the tube was inserted into the right jugular vein and tied. The proximal side of the left carotid artery was clamped to block the blood flow temporarily, while the free end of the tube was inserted into the artery and tied. The clamp was released and the blood flow through the tube was confirmed. After 20 min, the silk thread was removed from the tube and its wet weight was immediately measured. The dry weight was measured after 20 min at 60°C. The amount of thrombus formed was defined as the wet weight or as the dry weight minus the weight of the silk thread.

Inferior vena ligation model in rats

The method employed here followed Wessler[21] with slight modification.[22,23] The rats' grouping, dosage, anaesthesia and administration were as mentioned above. After the administration, an incision was made in the medioventral line, the inferior vena of the rats was separated and then ligated closely under the left kidney vein. The operation was finished in 5 min. After 3 h, abdominal walls were opened again. We cut open the vessel wall longitudinally under the ligation and pulled out the thrombi in the vein; their wet weight or dry weight was measured as mentioned above. Meanwhile, 3 ml blood was drawn from the carotid artery and then centrifuged at 3000 rpm for 15 min at room temperature. Plasma t-PA/PAI assay was performed as instructed in the ELISA kit.

Protection on endothelia analysis

The rats' grouping, dosage, anaesthesia, administration and model establishing were as for the inferior vena ligation model in rats. After the operation, the ligation lasted for 24 h, following which the vessel undergoing thrombosis was removed and immersed in 4% glutaraldehyde, processed for transmission electron microscopy (TEM) and, finally, the samples were sent to the TEM centre of China Pharmaceutical University.

Anti-platelet aggregation assay

Blood was withdrawn from the carotid artery of male New Zealand white rabbits after local anaesthesia with lidocaine and directly collected into citrated (1 : 10 dilution, 3.8% sodium citrate) tubes. The blood sample was centrifuged at 1000 or 1600 rpm for 15 min at room temperature to prepare platelet-rich plasma (PRP) or platelet-poor plasma (PPP), respectively.

Platelet aggregation was measured by using an aggregometer (Beijing Steellex Science Instrument Company, China) according to the turbidimetry method of Born and Cross[24] as previously described by Jin *et al.*[25] Briefly, 250 μl PRP was incubated at 37°C in the aggregometer with stirring at 1000 rpm and then 10 μl physiological saline, aspirin or various concentrations of pENW was added. After 5 min preincubation, platelet aggregation was induced by the addition of ADP (12.5 μM). Maximal platelet aggregation (MPA) was defined as the maximal per cent increase in light transmission after addition of ADP related to the light transmission in PPP. The maximal gradient of platelet aggregation was defined as the maximal per cent increase within the first 5 min.

Statistical analysis

The experimental results were expressed as the mean ± SD for ten in each group. Data were assessed by analysis of variance. If this analysis indicated significant differences between the group means, then each group was compared with control group by using the Dunnett t (2-sided), and $P < 0.05$ was considered to be statistically significant.

Results

Coagulation time and haemorrhage time

The coagulation time was markedly increased, in a dose-dependent manner, in mice treated with pENW (Figure 2). On the other hand, Figure 3 shows that the haemorrhage time was not increased, but the positive drug, low-molecular-weight heparin calcium, can significantly increase it. The difference between LMWHCa and pENW (8, 4 mg/kg) is statistically significant.

Inhibition of thrombi formation

Compared with the saline-treated group, both the wet and dry weight of thrombus was markedly decreased, in a dose-dependent manner, in the arterio-venous shunt model and inferior vena ligation model in pENW-treated rats (Figures 4, 5). Positive drugs, aspirin and urokinase, also significantly decreased it.

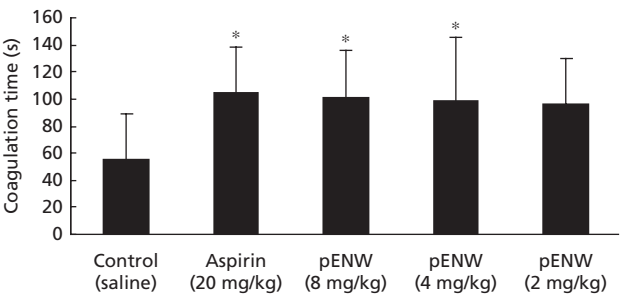

Figure 2 The effect of pENW on the coagulation time in mice. Data are presented as mean ± SD, $n = 10$. *$P < 0.05$ vs saline-treated group.

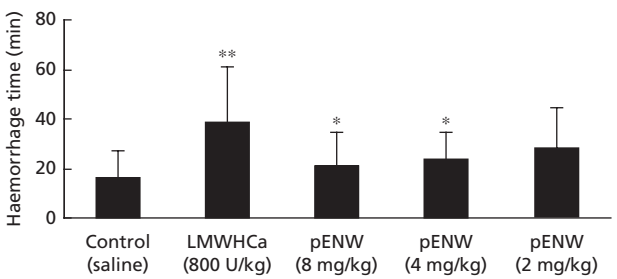

Figure 3 The effect of pENW on the haemorrhage time after tail cutting in mice. Data are presented as mean ± SD, $n = 10$. *$P < 0.05$ vs positive control group; **$P < 0.01$ vs saline-treated group.

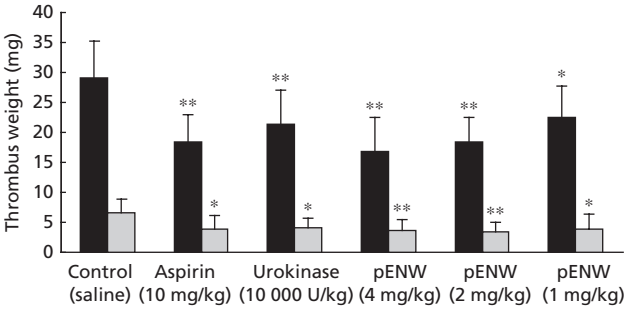

Figure 4 The effect of pENW on the thrombus weights in the arterio-venous shunt model in rats. Black columns, wet weight; grey columns, dry weight. Data are presented as mean ± SD, $n = 10$. $^{**}P < 0.01$, $^{*}P < 0.05$ vs saline-treated group.

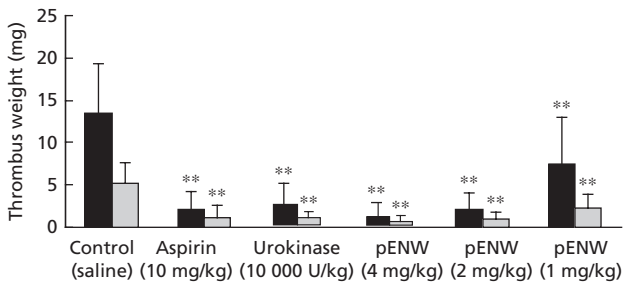

Figure 5 The effect of pENW on the thrombus weights in inferior vena ligation model in rats. Black columns, wet weight; grey columns, dry weight. Data are presented as mean ± SD, $n = 10$. $^{**}P < 0.01$, vs saline-treated group.

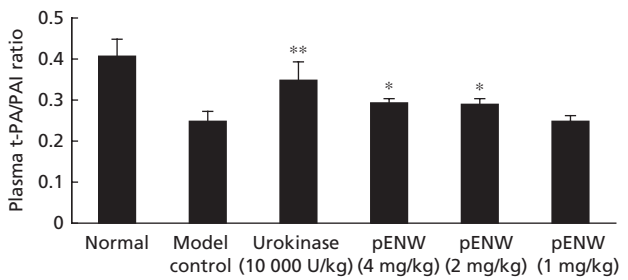

Figure 6 The effect of pENW on the ratio of plasma t-PA/PAI in rats. Data are presented as mean ± SD, $n = 10$. $^{**}P < 0.01$, $^{*}P < 0.05$ vs model control group.

Effect of pENW on the plasma fibrinolysis system

pENW-treated rats, as well as urokinase-treated rats, exhibited a significantly increased ratio of plasma t-PA/PAI (Figure 6), which is a useful indicator of fibrinolytic balance. The elevation of the ratio means pENW can enhance fibrinolytic activity when it is used in thrombosis.

Ultrastructure changes of vascular endothelium

The ultrastructure of endothelium in vessels developing thrombosis, including rough endoplasmic reticulum (RER) dilating, vascular endothelium detaching from basement membrane, cell membrane disappearing, surface polysaccharide vanishing, lumens filled up with plenty of erythrocytes which interlace endothelium, obviously deviated from normal (Figure 7). In the rats treated with pENW, the endothelium showed a better condition, being slightly separated from basement membrane, which was nearly integrated, mitochondria partly damaged, and few erythrocytes in the lumens. The protective action of pENW was also concentration dependent. This indicated that pENW can ameliorate the endothelial damage induced by thrombosis.

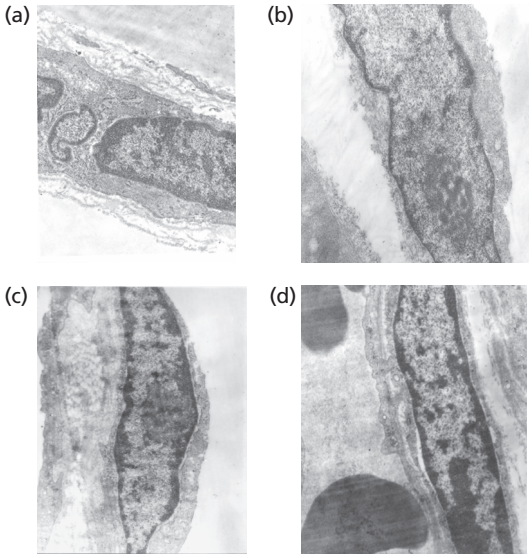

Figure 7 The protective action of pENW on the endothelial damage induced by thrombosis in rats. (a) Normal rats: well-distributed chromatin, elliptic nucleus, normal nuclear membrane, normal morphous of cytoplasmic organelles, pappo-like polysaccharide on the surface of and between cells, integrated and uniform basement membrane. (b) Model rats: rough endoplasmic reticulum dilating, vascular endothelium detaching from basement membrane, cell membrane disappearing, surface polysaccharide vanishing, lumens filled up with plenty of erythrocytes which interlace endothelium. (c) Rats treated with 4 mg/kg pENW: well-distributed chromatin, endothelium slightly separated from basement membrane, which is nearly integrated, mitochondria partly damaged and few erythrocytes in the lumens. (d) Rats treated with 1 mg/kg pENW: endothelium separated from basement membrane, which is partly dissolved, mitochondria exhibit vacuolation and some erythrocytes in the lumens. (Hitachi, H7000 Transmission Electron Microscope).

Effect of pENW on platelet aggregation

pENW and aspirin significantly decreased platelet aggregation induced by ADP in rabbits. pENW elicited a concentration-dependent decrease of platelet aggregation induced by ADP (Figure 8).

Discussion

Over the last several decades, research on snake venom toxins has inspired the design and development of a number of therapeutic agents. Blood circulation, particularly thrombosis and haemostasis, is one of the major targets of several snake venom proteins. Important progress has been made in understanding the relationship between blood coagulation-associated factors and anticoagulant proteins from snake

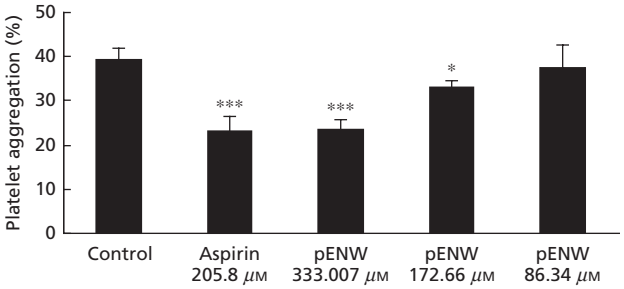

Figure 8 The effect of pENW on platelet aggregation induced by ADP in rabbits. Data are presented as mean ± SD, $n = 4$. ***$P < 0.001$, *$P < 0.05$ vs model control group.

venoms.[26–28] Such studies contribute to our fight against unwanted clot formation that leads to aggravation, or even death in cardiac arrest, and stroke in patients with cardiovascular and cerebrovascular diseases, arteriosclerosis and hypertension. However, we regrettably found that treatment and prevention always go along with higher risk. There has yet to be found a peptide or protein that can prevent coagulation without promoting bleeding, and that is why pENW first attracted our attention. As we know, the severe side effect of bleeding excludes a large number of patients. Consequently, the fact that pENW could prolong the coagulation time while not extending the haemorrhage time grants it another outstanding point in addition to its small molecular weight and stable nature.

The antithrombotic activity of pENW provided us with another surprise. There are two different kinds of thrombosis – arterial thrombosis, a frequent consequence of atherosclerosis, which can occur in regions of moderate to high shear stress through adhesion and aggregation of platelets at the luminal surface of damaged vessels and may lead to their occlusion, and venous thrombosis, which is associated with blood coagulation, and is a pathological condition appearing in post-traumatic and postoperative periods as deposits of fibrin and erythrocytes in regions of stasis or low shear stress.[1] To aim directly at the former, we employed an arterio-venous shunt model, in which the thrombus is primarily composed of platelets, and in which antiplatelet drugs or thrombolytic drugs have effect.[20] As for the latter, an inferior vena ligation model was used, in which stasis and hypercoagulability are the main courses, and in which anticoagulants or thrombolytic drugs would have effect.[21] According to the insignificant effect of pENW on the haemorrahge time and the positive results on two thrombus models with different pathogenesis, either anticoagulation or antiplatelet activity could wholly explain the mechanism. As a result, we tested its direct action of thrombolysis; the negative result indicates that its antithrombotic activity lies in some underlying effect.

Many components of the fibrinolytic system, including plasminogen and α^2-antiplasmin, are produced by the liver parenchyma. Since plasminogen and α^2-antiplasmin are abundantly present in the blood at rather stable levels, the blood fibrinolytic activity is mainly determined by the balance between t-PA, which directly converts plasminogen to plasmin, and PAI-1, which rapidly forms a stable inactive complex with t-PA. Vascular endothelial cells synthesize and secrete t-PA and are considered to be the predominant source of t-PA in the circulation.[29] The endothelial cells possess binding sites with high specificity and affinity both for t-PA and for plasminogen. Thus plasminogen can be converted to plasmin on the vascular surface. This process considerably enhances the fibrinolytic activity at the blood vessel wall. The elevation of plasma t-PA/PAI in pENW-treated rats with thrombosis might be related to the vascular endothelium.

With respect to the essential role of endothelium on thrombosis and haemostasis, the protective action of pENW on vascular endothelium is of great significance. In view of the TEM detection report, pENW could interfere with adhesion of erythrocytes to vascular endothelium. It has been reported that alterations in erythrocyte membrane, such as phosphatidylserine (PS) externalization and PS-bearing microvesicle generation, can enable erythrocytes to participate in blood coagulation and cell adhesion, which is correlated strongly with the presence and extent of thrombosis.[30,31] As a result, we are planning to test the effect of pENW on PS externalization on erythrocyte membranes induced by thrombosis. In addition, we will investigate the effect of pENW on production and elimination of endothelium-associated factors, such as nitric oxide (NO) for example, and try to investigate its effect in a further and exact manner.

According to the results of this study and the structure of pENW, its relationship with molecules on platelets, especially platelet glycoprotein IIb/IIIa complex (GPIIb/IIIa), is another target that needs to be investigated. It is reported that the binding of GPIIb/IIIa to ligands is ionic-bond-mediated, in a bivalent-cation-dependent manner.[32] Despite the structural diversity of GPIIb/IIIa ligands, they have in common an exposed aspartic acid or glutamic acid residue, critical for recognition by GPIIb/IIIa.[33] pENW is composed of three amino acids, with pGlu and Trp on either side and Asn in the centre. Asn is probably hydrolysed and transformed into Asp, which could subsequently bind cations. pGlu is made from dehydrated Glu, with electronegativity attenuated and hydrophobicity enhanced. Trp, with a benzene ring in the molecule, is fairly hydrophobic. It has been reported that RGDW ((Arg)-Gly-Asp-Trp)) has roughly a 200-fold higher affinity for GPIIb/IIIa than RGDS ((Arg)-Gly-Asp-Ser)), with respect to the ability of the peptide to inhibit platelet aggregation induced by ADP or thrombin.[34,35] This could be explained the fact that adding a more hydrophobic amino acid could enhance the hydrophobic interaction between GPIIb/IIIa and its ligands, consequently elevating the bonding force and activity. In addition, pENW is a small molecule consisting of only three amino acids, resulting in small stereospecific blockade, which means more drug molecules bind to the ligand-binding site in GPIIb/IIIa with high performance. Based on the theoretical presumption above, we will continue to study the interaction between platelet GPIIb/IIIa and pENW, which is of great value in developing an effective new drug in prevention and treatment of thrombosis.

Conclusions

In this study, the experimental results suggest that pENW could prolong the coagulation time in mice, without extending the haemorrhage time in the same way. It exerts obvious inhibitory actions on platelet aggregation and

thrombus formation in both arterial and venous thrombosis models, partly by interacting with vascular endothelium and interfering with adhesion of erythrocytes to endothelium. It has the potential to solve the problems of current drugs, principally to issues regarding safety and convenience when they are used clinically, and could be a promising drug for the prevention and treatment of thrombotic diseases with a good benefit-to-risk ratio. We are now investigating the molecular mechanisms of these beneficial effects of pENW on thrombotic disease, mainly on platelets and endothelium.

Conflict of interest

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

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