Antithrombotic Efficacy of Recombinant Tick Anticoagulant Peptide
A Potent Inhibitor of Coagulation Factor Xa in a Primate Model of Arterial Thrombosis

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Background. Tick anticoagulant peptide is a specific, potent inhibitor of blood coagulation factor Xa. The effects of recombinant tick anticoagulant peptide (rTAP) and standard heparin (SH) were compared in an anesthetized baboon model of arterial thrombosis where platelet deposition onto a Dacron vascular graft segment of an arteriovenous (AV) shunt was studied.

Methods and Results. Animals were randomized to receive systemic administration of SH (10 or 100 U/kg i.v. bolus followed by 0.4 or 1.0 U/kg/min i.v. infusion, respectively) or rTAP (6.25, 12.5, or 25.0 μg/kg/min i.v. infusion). rTAP, but not SH, caused a significant (p<0.05), dose-dependent reduction of indium-111 labeled platelet and iodine-125 labeled fibrinogen deposition onto the graft. Deposition was not significantly increased from baseline values during infusion of 12.5 or 25.0 μg/kg/min of rTAP. Blood flow was maintained at 64±9, 95±2, or 97±2% of baseline following infusion of 6.25, 12.5, or 25.0 μg/kg/min of rTAP, respectively. Both SH and rTAP significantly (p<0.05) decreased the systemic fibrinopeptide A (FPA) elevation during exposure to the Dacron graft. rTAP was fully antithrombotic at APTT values of 42.6±2.4 seconds (less than twofold basal value), while SH had no antithrombotic efficacy despite APTT values greater than 150 seconds (greater than fivefold basal value).

Conclusions. The demonstrated antithrombotic effect of rTAP in the absence of alterations in primary hemostasis suggests that controlling thrombin generation through inhibition of factor Xa may be a novel and effective pharmacological approach in the prevention of high-shear arterial thrombosis. ( Circulation 1991;84:1741-1748)

Normal hemostasis of the arterial vasculature requires carefully regulated interactions between the vessel wall and blood constituents. A disruption of the integrity of the vascular endothelial surface can result in occlusive thrombus formation. The morbidity and mortality resulting from the clinical manifestations of thrombi, such as acute myocardial infarction and cerebrovascular ischemia, have stimulated the search for pharmacologically effective antithrombotic agents.

Arterial thrombus formation is initiated by platelet adherence to the damaged subendothelium, platelet activation, and fibrinogen-mediated platelet aggregation. The exposure of the subendothelial surface results in activation of the coagulation cascade, primarily through the extrinsic pathway, with subsequent formation of factor Xa, Assembly of factor Xa, factor Va, and prothrombin into the prothrombinase complex on the surface of activated platelets results in local thrombin generation within the developing thrombus. Thrombin stabilizes the thrombus and promotes further growth through direct generation of fibrin, activation of factor XIII, and stimulation of platelet aggregation. Additionally, thrombin stimulates its own production by activation of factors V and VIII, resulting in an increase of clot-bound prothrombinase and tenase activity, respectively.

The critical role of thrombin in arterial thrombogenesis makes it an attractive target for pharmacological intervention. However, the clinical efficacy of standard heparin (SH) as an antithrombotic agent in the prevention and treatment of arterial thrombosis remains controversial. The reason for the ineffectiveness of SH as an antithrombotic agent is unclear, although inaccessibility of the complex to fibrin-bound thrombin, as well as the susceptibility of this complex to natural inhibitors has been implicated.

To overcome these limitations of heparin, numerous selective and potent, low molecular weight syn-
thmetic and natural inhibitors of thrombin have been developed. The improved efficacy of these inhibitors in animal models of thrombosis has been attributed to their enhanced accessibility to fibrin-bound thrombin and resistance to natural inhibitors. Despite the improved antithrombotic profile of thrombin inhibitors, their efficacy in arterial thrombosis models can be coincident with impairment of primary hemostasis, as evidenced by the prolongation of bleeding times.

Recently, natural and recombinant forms of activated protein C (APC) have been shown to inhibit platelet deposition in a baboon model of acute arterial thrombosis. The antithrombotic effect of APC was not associated with increased template bleeding time, presumably due to APC-induced inactivation of the non-enzymatic co-factors VIIIa and Va which resulted in a downregulation of thrombin formation.

In this study, we describe the effects of a potent and selective inhibitor of factor Xa in a baboon model of acute arterial thrombosis that has been used extensively to evaluate a variety of antithrombotic agents. The factor Xa inhibitor, tick anticoagulant peptide (TAP), is a recombinant form of a 60-amino acid polypeptide originally isolated from the tick Ornithodoros moubata. The selectivity and potency of TAP makes it an ideal tool to investigate the effects of directly inhibiting factor Xa under conditions of high shear arterial thrombus formation.

Methods

Test Compounds

Recombinant tick anticoagulant peptide (rTAP) was purified to homogeneity from a culture media of *Saccharomyces cerevisiae* as previously described. A single lot of recombinant inhibitor was used for the experiments. For study, rTAP was dissolved in sterile saline and infused intravenously at 6.25, 12.5, or 25.0 μg/kg/min at 0.58 ml/min.

Heparin sulfate (Upjohn, Kalamazoo, Mich.) was diluted in sterile saline and administered intravenously as an initial bolus followed by an infusion at doses of 10 U/kg bolus plus 0.4 U/kg/min (low dose) or 100 U/kg bolus plus 1.0 U/kg/min (high dose). The low dose of SH was selected to match the ex vivo APTT elevation obtained with the highest dose of rTAP as determined from preliminary experiments.

Experimental Protocol

Ten normal male baboons (*Papio anubis*), weighing between 18–25 kg, were used in this study. They were fasted overnight, sedated with ketamine hydrochloride (10 mg/kg i.m.) and acepromazine maleate (0.1 mg/kg i.m.), intubated, and maintained on 2% isoflurane (Anaquest, Madison, Wis.) for the duration of the experiment. A silastic femoral arteriovenous (AV) shunt was aseptically placed at least 2 hours before the study commenced. A 5-cm Dacron vascular graft segment (Bard Cardiosurgery, Billerica, Mass.) was inserted into the shunt to provide the thrombogenic stimulus. The shunt and vascular graft were prepared as described by Hanson et al.

Autologous platelets were labeled with 111In oxine (Amersham, UK). Briefly, the platelets were isolated from whole blood by differential centrifugation. The platelet-poor plasma was decanted and the platelet pellet was resuspended in 0.9% sterile saline. 111In oxine was added and free 111In was removed by gentle washing with saline. The labeled platelets were resuspended in autologous plasma and injected intravenously 1 hour prior to insertion of the first Dacron graft. Labeling efficiency was greater than 90% in all studies. Deposition of 111In-labeled platelets onto the thrombogenic surface was monitored using an NaI probe (Bicron, Meridan, Conn.) and a multichannel analyzer (Tracor, Chicago, Ill.) by obtaining 1 minute acquisitions of the 173 KeV peak at 5-minute intervals. The accumulation of platelets onto the graft segment was reported as the fold increase of 111In activity from baseline. Baseline values were determined using a 1-minute acquisition of a blood-volume matched segment of the silastic AV shunt prior to insertion of the Dacron graft.

Homologous fibrinogen was isolated from citrated plasma using β-alanine precipitation technique as described and labeled with 125I using Iodogen. Clottability of the labeled preparation was greater than 85% in all studies. Deposition of 125I-labeled fibrinogen onto the graft segment was determined using the 35 KeV photopeak of the 125I radionuclide and correcting for the 111In photopeak spillover in each study. The fold-increase from baseline was reported.

Blood flow through the AV shunt was monitored continuously during each experiment using a Doppler Ultrasonic flowmeter (Baylor College of Medicine, Houston, Tex.) and transducer probe positioned on the silastic tubing of the shunt. Flow was displayed on a Hewlett-Packard recorder (Chelmsford, Mass.). Initial blood flow through the shunt ranged from 150–210 ml per minute. Blood flow was reported as the percent of baseline flow.

The baboons were randomized to the following treatment groups: Saline control (n=4), SH (10 U/kg i.v. bolus followed by 0.4 U/kg/min; n=4), SH (100 U/kg i.v. bolus followed by 1.0 U/kg/min; n=4), rTAP (6.25 μg/kg/min; n=4), rTAP (12.5 μg/kg/min; n=4), or rTAP (25.0 μg/kg/min; n=4). The test compounds were administered systemically through a cephalic vein catheter. In each study, two Dacron vascular grafts were sequentially inserted into the AV shunt of each baboon. In all experiments, saline was infused during exposure to the first graft; thus, the thrombotic response to the first graft segment served as an internal control for each baboon. Following occlusion of the initial Dacron graft, the segment was removed and the AV shunt was reestablished. The appropriate therapy corresponding to the treatment group was infused for 1 hour prior to the 60-minute exposure to the second graft. Following occlusion, or 60 minutes after
insertion of the second graft if the Dacron graft did not occlude, the silastic shunt was removed and the baboon was allowed to recover. A period of 4 to 6 weeks was allowed before restudy of individual baboons. All procedures were reviewed and approved by the Institutional Committee for the Care and Use of Laboratory Animals and complied with Federal regulations.

**Laboratory Studies**

Whole blood platelet and red and white cell counts were determined using a Baker system 9000 Counter (Baker, Allentown, Pa.) with EDTA-anticoagulated blood. Ex vivo aggregation in response to adenosine diphosphate (20 µm) and collagen (10 µg/ml) was studied using platelet-rich plasma adjusted to 2.5×10⁴ platelets/ml with time-matched platelet-poor plasma. Responses were studied using an aggregometer and chart recorder (Chrono-Log, Havertown, Pa.). Fibrinogen concentration and APTT were determined using a Coag-A-Mate XC (General Diagnostics, Durham, N.C.) and standard reagents (General Diagnostics). Fibrinopeptide A (FPA) values were determined using a radioimmunoassay (ByK-Sangtec Diagnostica, Dietzenbach, FRG). Template bleeding times were performed using a Simplate® device (Organon Teknika, Durham, N.C.) on the shaved forearm. The plasma concentration of rTAP was determined as previously detailed.

**Statistical Analysis**

Data shown represent mean±SEM. The effect of rTAP or SH on ¹¹¹In-labeled platelet deposition and ¹²³I-labeled fibrin(ogen) deposition was studied using the fold-increase from baseline. The effect of rTAP or SH on blood flow was determined by analyzing the absolute change. The statistical analyses were performed on changes in response during treatment infusion (graft 2) versus saline infusion (graft 1) in each animal using repeated measures analysis of variance. Differences from saline response at each time point were determined using Dunnett's test (one sided, p<0.05). Dose-related responses to rTAP and SH were determined using linear contrasts of the time-response means. A two-sided paired Student's t test was used to study differences between graft 1 and graft 2 on the following parameters: activated partial thromboplastin time, bleeding time, thrombus weight, and FPA values.

**Results**

**Thrombus Formation in Control Grafts**

In each study, two Dacron graft segments were sequentially placed in a single baboon. The initial vascular graft, which served as the control, was inserted into the AV shunt during infusion of saline. In all studies (n=24), this first graft segment occluded within 60 minutes (38±2 minutes). After 30 minutes of exposure to the initial graft, ¹¹¹In-labeled platelet and ¹²³I-labeled fibrin(ogen) deposition onto the segment increased by 11.19±0.85- and 2.51±0.28-fold baseline, respectively. This ratio of platelet/fibrin-(ogen), as well as the histopathology of the segment, reflected the platelet-rich composition of the thrombus. Systemic FPA increased from 3.16±0.40 to 21.40±1.70 ng/ml during exposure to the Dacron graft, documenting systemic thrombin-mediated generation of fibrin in this model. Following occlusion, the initial Dacron graft segment was removed, and blood flow through the AV shunt was reestablished. The appropriate therapy (saline, rTAP, or SH) was infused systemically for one hour prior and during exposure to the second graft segment. In the control group (saline infusion during first and second Dacron graft segment exposure), ¹¹¹In-labeled platelet and ¹²³I-labeled fibrin(ogen) deposition were not significantly different between the first and second graft segments (Figure 1A). Additionally, the time to occlusion and rate of blood flow reduction were not signif-
Effects of basal rTAP resulted in a significant (p<0.05), dose-dependent decrease of 111In-labeled platelet deposition onto the Dacron graft segment (Figure 2A). Compared with the saline-treated graft 1 segment, rTAP inhibited platelet deposition by 33±12, 83±4, or 84±2% at 6.25, 12.5, or 25.0 μg/kg/min, respectively. Deposition of 125I-labeled fibrinogen was also significantly (p<0.05) decreased compared with controls in a dose-dependent manner by 13±20% (6.25 μg/kg/min), 57±1% (12.5 μg/kg/min), and 63±10% (25.0 μg/kg/min) during rTAP infusion. None of the AV shunts in baboons treated with rTAP occluded during the 60-minute study period. Blood flow was maintained at 64±9% (6.25 μg/kg/min), 95±2% (12.5 μg/kg/min), or 97±2% (25.0 μg/kg/min) of baseline value at 60 minutes in animals infused with rTAP (Figure 2B). The antithrombotic effects of rTAP were consistent with its plasma concentration (Figure 2C). Consistent with the effects on platelet and fibrinogen deposition, rTAP dose-dependently decreased (r=0.87, p<0.05) thrombus weight (Figure 4A). At the higher doses of rTAP (12.5 and 25.0 μg/kg/min), the poststudy weight of the Dacron graft segment was equivalent to that of a blood-saturated graft, confirming the lack of thrombus formation.

In contrast to the profound effect of rTAP on thrombus formation, neither dose of SH evaluated significantly reduced the extent of platelet (Figure 3A) deposition compared with control grafts in this baboon model. However, the average time to occlusion of the AV shunt in animals treated with SH was significantly (p<0.05) increased in a dose-related manner. One of four AV shunts and three of four AV shunts did not occlude during infusion of the low or high dose, respectively, of SH. Blood flow was, however, significantly (p<0.05) decreased at 60 minutes to 20±20% (low dose SH) and 35±21% (high dose SH) of baseline value (Figure 3B). SH decreased thrombus weight in only one of four animals at each dose level (Figure 4B) so that there was no significant relation between (r=0.30, p<0.05) thrombus weight and SH dose.

Effects of rTAP and SH on In Vivo and Ex Vivo Parameters of Hemostasis

The effect of rTAP and SH on bleeding time and APTT and FPA values are shown in Table 1. Administration of rTAP resulted in a significant (p<0.05), dose-dependent prolongation of APTT; however, full antithrombotic efficacy was obtained at a dose of rTAP (12.5 μg/kg/min) which minimally (<twofold basal value) increased APTT. In contrast, SH had no significant effect on platelet deposition at doses which elevated APTT by approximately twofold (low dose SH) or more than fivefold (high dose SH) basal value.

Placement of the initial Dacron graft segment significantly (p<0.05) elevated FPA from baseline values in all studies. All doses of rTAP studied prevented the systemic FPA response during exposure to the second graft segment. SH dose-dependently inhibited systemic FPA. The high dose of SH caused similar inhibition of FPA as was obtained
with rTAP; however, this dose of SH had no effect on 111In-labeled platelet deposition. Neither rTAP or SH administration significantly increased template bleeding times in this study.

Administration of rTAP or SH had no significant effect on platelet, red or white cell counts, fibrinogen concentration, or ex vivo platelet aggregation in response to ADP or collagen at the doses evaluated in this study.

**Discussion**

Tick anticoagulant peptide TAP, which was originally isolated from the soft tick *Omrithodoros moubata* and subsequently made recombinantly in yeast (rTAP), has been characterized as an extremely potent and selective inhibitor of blood coagulation factor Xa (FXa). These properties of rTAP make it a unique and valuable tool to study the in vivo antithrombotic and antithrombotic effects of selective FXa inhibition. Previously rTAP has been shown to effectively prevent venous thrombus formation in rabbits as well as suppress systemic elevations in FPA induced by intravenous administration of thromboplastin in conscious Rhesus monkeys. In this report we describe the effects of rTAP in a baboon model of high-shear, platelet-dependent thrombus formation which has been used extensively to evaluate the antithrombotic potential of a number of agents. In this model, a Dacron vascular graft segment of an AV shunt provided the thrombogenic stimulus for platelet and fibrin(ogen) deposition. Antithrombotic efficacy was assessed by monitoring the deposition of 111In-labeled platelets and 125I fibrin(ogen) onto the Dacron surface, as well as blood flow through the AV shunt.

rTAP dose-dependently inhibited platelet and fibrin(ogen) deposition onto the Dacron graft segment. Correspondingly, rTAP prevented occlusion of the graft segment at all doses studied and also prevented the decreases in blood flow associated with platelet deposition at doses of 12.5 and 25.0 µg/kg/
TABLE 1. Effect of rTAP or Standard Heparin on Hemostatic Parameters in Baboons

<table>
<thead>
<tr>
<th></th>
<th>Bleeding time (min)</th>
<th>APTT (seconds)</th>
<th>FPA (ng/ml)</th>
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<tbody>
<tr>
<td></td>
<td>PG</td>
<td>G1</td>
<td>G2</td>
</tr>
<tr>
<td>rTAP 6.25</td>
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<td>1.9±0.1</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>rTAP 12.5</td>
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<td>2.4±0.2</td>
<td>2.8±0.1</td>
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<tr>
<td>rTAP 25.0</td>
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<td>2.0±0.4</td>
<td>2.8±0.3</td>
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<tr>
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<td>1.9±0.1</td>
<td>2.0±0.4</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>SH high</td>
<td>2.1±0.2</td>
<td>2.3±0.1</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2.0±0.2</td>
<td>2.3±0.1</td>
<td>2.5±0.2</td>
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</tbody>
</table>

PG, pre-Dacron graft exposure; G1, control graft (saline); G2, treatment group graft; rTAP 6.25, 6.25 μg/kg/min i.v.; rTAP; rTAP 12.5, 12.5 μg/kg/min i.v.; rTAP; rTAP 25.0, 25.0 μg/kg/min i.v.; rTAP; SH, standard heparin 10 U/kg i.v. bolus followed by 0.4 U/kg/min; SH, standard heparin 100 U/kg i.v. bolus followed by 1.0 U/kg/min.

Values represent mean±SEM (n=4/treatment group).

Samples for PG were obtained at 10 minutes prior to insertion of and G1 at 20 minutes postinsertion of G1 and G2.

*Significantly (p<0.05) different from baseline.

The antithrombotic effect of rTAP was associated with modest elevations in ex vivo APTT and insignificant effects on template bleeding time. The potent antithrombotic effect of rTAP was in sharp contrast to SH, which had no significant effect on platelet deposition at doses which elevated ex vivo APTT to greater than fivefold over basal level. These results are consistent with previous studies which have demonstrated that platelet-rich, high-shear arterial thrombus formation is resistant to conventional doses of SH.4,12,23,45,46 The poor antithrombotic profile of SH has been attributed to numerous factors which include neutralization by endogenous inhibitors, and restricted access of the heparin antithrombin-III (AT-III) inhibitory complex (SH/AT-III) to fibrin-bound thrombin and FXa within the developing thrombus.18-20 Compared with SH, low molecular weight heparanoids (LMWH) have been shown to have more favorable antihemostatic effects at comparable antithrombotic doses in models of high-shear, platelet-dependent arterial thrombosis.12 However, due to the requirement of AT-III to form the inhibitory complex, LMWH's, like SH, remain poor antithrombotics in these models.

The observed elevations in systemic FPA levels following graft insertion in the AV shunt were effectively suppressed by both rTAP and SH. The seemingly paradoxical effects of SH on FPA generation and thrombus formation may reflect the different nature of the thrombin activity responsible for these events. The observed suppression of systemic FPA by SH may result from the rapid inactivation of soluble thrombin released at the site of the graft insertion by the SH/AT-III complex. In contrast, the inability of SH to effectively prevent thrombus formation localized to the graft, may reflect the inability of the SH/AT-III inhibitory complex to neutralize fibrin bound thrombin activity.18 The results obtained here are similar to the observed antithrombotic effects of direct (i.e., AT-III independent), low molecular weight inhibitors of thrombin such as hirudin and hirudin analogs, D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK), and (2R,4R)-4-methyl-l-[N2-(3-methyl-1,2,3,4-tetrahydro-8-quinoinesulfonyl)]-L-arginyl]-2-piperidinocarboxylic acid (Argatroban), in this model and others.4,15,23-25,27,29,30,47 These results strongly support the view that thrombin is the primary mediator of arterial thrombus formation. The potent effects of rTAP on systemic FPA elevations and thrombus formation are comparable with those observed for AT-III independent direct thrombin inhibitors in this model.4,30-32 This suggests that the inhibition of thrombin formation catalyzed by the prothrombinase complex is an effective means of controlling thrombin activity both systemically and locally at the site of thrombus formation.

The favorable antithrombotic effects of AT-III independent thrombin inhibitors,4,30 as well as anti-platelet agents34-36 in a similar model to that used here have been correlated with significant alterations in primary hemostasis, reflected in elevations of bleeding time. In contrast, there was no significant elevation in template bleeding time at fully antithrombotic doses of rTAP. In this regard, the effects of rTAP are similar to those reported for activated recombinant protein C (rAPC) in an analogous model, where there were insignificant elevations in bleeding time at doses of this protease which were antithrombotic.32 rAPC acts to inhibit thrombin activity principally through the proteolytic inactivation of the non-enzymatic co-factors, factor Va and factor VIIIa which results in a reduction of prothrombinase activity. It is currently unknown why rTAP and rAPC differ from other antithrombotic agents used in this model with respect to their effects on primary hemostasis. One explanation may be related to the rate of prothrombinase inactivation by both rTAP and rAPC. Slow inactivation of newly generated prothrombinase complexes by these agents might result in sufficient thrombin generation to adequately permit primary hemostatic plug formation but insufficient to support high-shear thrombus development.32

In contrast with the effect of a number of other agents used in this model including SH,4,12 hirudin,30 PPACK,4 and rAPC,32 the antithrombotic effects of
rTAP described here were not associated with large elevations of ex vivo APTT. The apparent lack of correlation between the ex vivo APTT and the antithrombotic effects of rTAP is primarily due to the nature of this clotting assay. The accessibility of soluble thrombin in this assay makes it a sensitive and reliable means to evaluate the anticoagulant effects of direct and indirect inhibitors of thrombin. The evaluation of direct inhibitors of FXa in this assay, however, is complicated by the fact that this enzyme catalyzes the formation of thrombin only following the assembly of the prothrombinase complex. Therefore, the accessibility of inhibitors to FXa within this complex, as well as the relative rate of association with the enzyme over the time course of the assay, determines the anticoagulant effect of an inhibitor. The accessibility of FXa in the prothrombinase complex to low molecular weight inhibitors such as rTAP may not be an important factor in the inhibition of prothrombinase activity; however, the interaction of this inhibitor with FXa is characterized by a kinetically slow rate of association.39 In an APTT assay, this relatively slow rate of FXa inhibition may allow enough thrombin to be generated to result in rapid clot formation which could be interpreted as a weak anticoagulant effect. Whereas the effect of rTAP is directly on prothrombinase activity, the anticoagulant effect of rAPC is a result of both direct and indirect effects on this catalytic complex through the selective inactivation of factors VIIIa and Va, respectively.32 The differences between rTAP and rAPC clearly illustrate that although a correlation between elevations in ex vivo APTT and the antithrombotic effects of an anticoagulant can be made, care should be taken when using this assay to predict and compare the antithrombotic effects of other anticoagulants.

In conclusion, the highly selective and potent FXa inhibitor rTAP has been shown to effectively inhibit platelet deposition without adversely affecting primary hemostasis in a primate model of high-shear, platelet-dependent thrombosis. The dramatic antithrombotic effects of rTAP in this model suggest that potent and essentially irreversible FXa inhibition may be therapeutically useful in the prevention of acute arterial thrombus formation.

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References


KEY WORDS • factor Xa inhibitor • baboon model of arterial thrombosis • anticoagulant peptide • heparin
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