International Journal of Pharma and Bio Sciences V1(1)2010

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

Shvetank Bhatt, Dilip K. Pandey* and K.S.R. Pai,††*

*Pharmacy Group, FD-III, Birla Institute of Technology and Science, Pilani, Rajasthan-333031 India
†† Manipal College of Pharmaceutical Sciences, Manipal, Karnataka-576104, India

Corresponding Author shvetankbhatt@gmail.com

Abstract

Thrombosis is one of the major causes of death worldwide. It occurs due to imbalance between pro-thrombotic and fibrinolytic pathway. The clot formation activates the endogenous fibrinolytic system, for conversion of plasminogen to plasmin. Plasmin degrades the fibrin clot, restoring blood flow to vital organs. Thus, fibrinolysis is a physiological mechanism designed to remove intravascular thrombi while maintaining vascular patency. Several fibrinolytic drugs such as Streptokinase, recombinant t-PA and Reteplase are used clinically, principally to reperfuse the occluded coronary artery in diseases such as angina pectoris. However, these thrombolytics have many shortcomings such enhanced risk of bleeding such as GI haemorrhage. Present study was carried out for evaluation of potato tuber carboxypeptidase inhibitor, which is an inhibitor of thrombin activatable fibrinolysis inhibitor (TAFI) a newer target for fibrinolytic drugs which inhibits the conversion of plasminogen to plasmin with other standard drugs like aspirin and clopidogrel in models of thrombososis and coagulation.

Key words: Thrombosis, potato tuber carboxypeptidase inhibitor, thrombin activatable fibrinolysis inhibitor, ferric chloride.

Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) is a Carboxypeptidase B like enzyme shows important role in regulation of fibrinolysis[1]. It is synthesized in liver and circulates in plasma (2.5µg/ml) as an inactive zymogen in a complex with plasminogen[2] and activated by trypsin, plasmin, & thrombin. TAFI is converted to active TAFIa by the action of thrombin-thrombomodulin complex[3]. Levels of TAFI are elevated in a variety of thrombotic diseases such as deep vein thrombosis and unstable angina[4, 5]. High levels of plasma TAFI have also been reported in other metabolic diseases that are associated with increased thrombotic risk, including obesity and non-insulin dependent diabetes mellitus (NIDDM) [6, 7].

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

Therefore, inhibition of TAFIa will provide an attractive profibrinolytic strategy. A 39-amino acid protein PTCI is naturally occurring in potatoes that can form complexes with several metallo-carboxypeptidases (including TAFI) and inhibiting them in a strong competitive way with a Ki in the nanomolar range[8].

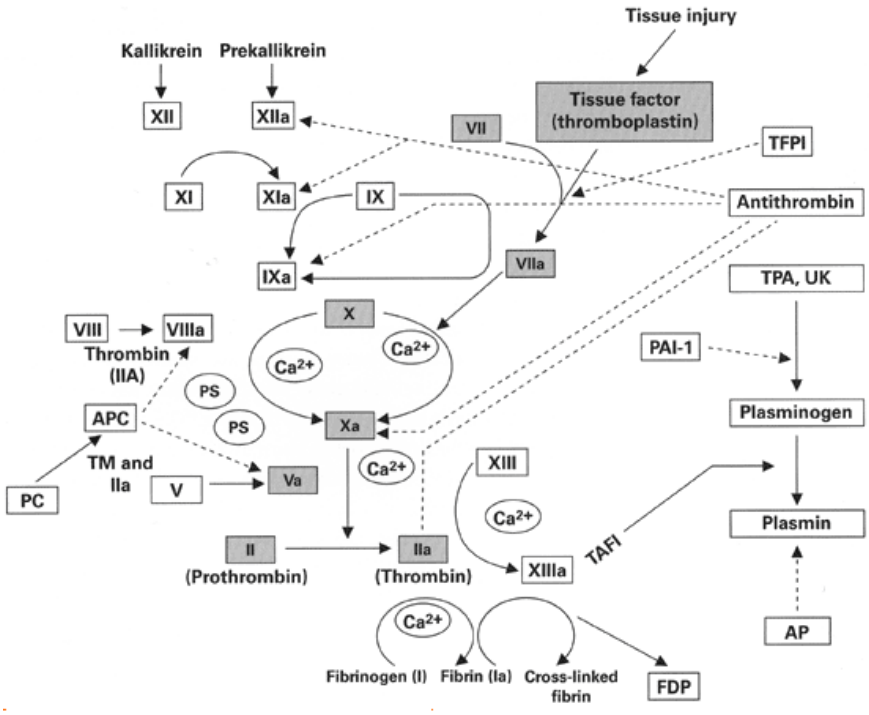

Figure 1

Showing effect of TAFI in coagulation pathway The tissue factor–dependent pathway is shown by gray shading. Dashed lines represent site of major inhibitors. AP, α-antiplasmin; APC, activated protein C; FDP, fibrin split products; PAI, plasminogen activator inhibitor; PC, protein C; PS, protein S; TAFI, thrombin-activatable fibrinolysis inhibitor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; TPA, tissue plasminogen activator; UK, urokinase.

Previous study suggested that, PTCI potentiates fibrinolysis by inhibiting TAFIa-dependent removal of C-terminal lysine residues exposed on fibrin partially degraded by plasmin[9]. Aspirin alters the balance between TXA_2, which promotes aggregation,and PGI_2 which inhibits it. Aspirin

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

inactivates cyclooxygenase acting mainly on the constitutive form of COX-1 by irreversibly acetylating a serine residue in its active site[10].This reduces both TXA_2 synthesis in platelets and PGI_2 synthesis in endothelium. Vascular endothelial cells however, can synthesize new enzyme whereas, platelets cannot. After administration of aspirin, TXA_2 synthesis does not recover until the affected cohort of platelets is replaced in 7-10 days. Clopidogrel inhibits ADP induced inhibition of adenylate cyclase, which leads to elevation of cAMP levels in the platelets. Elevated cAMP levels reduces platelet reactivity by decreasing agonist binding to platelet membrane receptors, by inhibiting the generation of activating signal molecules in the phosphoinositide pathway, reducing the intracellular calcium concentration by stimulating the sequestration of calcium into the dense tubular system and calcium efflux, and inhibiting myosin light chain kinase activity. By blocking the ADP receptor, clopidogrel inhibits the binding of fibrinogen to its platelet receptor, the glycoprotein GP IIb/IIIa[11, 12, 13]. The current study was undertaken to investigate the anti-thrombotic activity of PTCI in various model of thrombosis.

Materials and Methods

Animals

Eight- to ten-week-old male Wistar rats (250–300 g) were used in this study. The animals were kept in individually ventilated cages in a room with controlled temperature (23 ± 2 ° C), lighting (12: 12 h light-dark cycle) and relative humidity (55 ± 10%). Animals had free access to standard rat chow and water. The experiment was conducted in accordance with the protocol approved by Institutional animal ethical committee, Zydus Research Centre, India .

Chemical Reagents

PTCI and human recombinant thrombomodulin were purchased from Sigma Chemical, St. Louis, Mo., USA, and American Diagnostica, Stamford, Conn., USA, respectively. tPA (Actilyse) was procured from Boehringer Ingelheim, bovine thrombin from Hi Media, India clopidogrel and aspirin from zydus research centre Ahemdabad.

$FeCl_3$ induced thrombosis model in rats

$FeCl_3$ -induced chemical injury was used as a model of arterial thrombosis [14]. Rats were anaesthetized with urethane (1.25 g/ kg, i.p.) and secured in supine position. A midline cervical incision was made on the ventral side of the neck, and left carotid artery was isolated by blunt dissection. A 2*3 mm strip of Whatman filter paper No. 1 saturated with 35% (w/v) $FeCl_3$ was placed on the carotid artery for 5 min. A temperature probe (Thermalert-TH8, Physitemp Instruments Inc., Clifton, N.J., USA) was placed distal to filter paper to monitor the temperature of carotid artery. A sudden decrease in temperature (about 2 ° C) was taken as an indication of cessation of blood flow as a consequence to thrombus formation. Time to occlusion (TTO) was defined as the time from $FeCl_3$ application to time of thrombus formation[15, 16]. A cutoff time was fixed at 60 min in case no thrombus formation was seen in drug-treated animals. The study design for $FeCl_3$ -induced arterial thrombosis included groups of animals. Group one (n = 6) received saline i.v. 30 min prior to $FeCl_3$ application. PTCI was dissolved in saline for i.v. administration. Group two (n = 6/dose) was administered PTCI (0.5, 1, 2, 4 mg/kg, i.v.) 30 min. Group three (n=6/dose) received aspirin (5, 10, 25, 50 mg/kg, po) as methyl cellulose suspension (0.5%) and group-4 (n=6/dose) received clopidogrel (3, 5, 10 mg/kg, po) dissolved in saline

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

at 30 min, 90 min and 120 min respectively prior to $FeCl_3$ application

Bleeding Time Using Tail Transection Test

Male Wistar rats were anaesthetized with urethane (1.25 g/kg, i.p.) and their tails were cleaned with saline. Tails were transected 2 mm from the tip with a sterile surgical scalpel blade and immediately immersed in a test tube filled with normal saline maintained at 37 ° C. Bleeding time was assessed as the time from the transaction of tail tip to the cessation of blood flow[17]. In the case the bleeding did not stop, a maximum cutoff bleeding time was fixed at 30 min. This study included three groups of animals. Group one (n = 6), received vehicle i.v. 30 min prior to tail bleeding test. Group two (n = 6) was administered PTCI (4 mg/kg, i.v.) 30 min prior to tail bleeding test. Group three (n = 6) received aspirin (200 mg/kg, po) 90 min prior to tail bleeding test and Group four (n = 6) received clopidogrel (10 mg/kg, po) 120 min prior to tail bleeding test.

Activated partial thromboplastin time (APTT) determination

Drugs (aspirin, 200 mg/kg po, clopidogrel, 10 mg/kg po and PTCI 4 mg/kg iv) were administered 18 – 20 hours fasted male male Wistar rats (n=6). After 2 hours of drug administration blood samples were collected from anaesthetized rat (pentobarbital, 45 mg/kg, i..p), into a 1ml-microfuge tubes containing an anticoagulant sodium citrate solution (3.8%) in (9:1) ratio. Centrifuge the citrated blood samples at 1500g for 15 min at room temperature, Supernatant plasma was carefully transferred into other microfuge tube for estimation by turbidity method. The thrombin-catalyzed conversion of fibrinogen to fibrin is the final reaction in the 'coagulation cascade'[18]. Fibrin formation results in an increase in turbidity, which is detected by photometer at 470ηm. Incubating the plasma with optimal amount of phospholipids and a surface activator activates factors of the intrinsic coagulation system. The addition of calcium ions triggers the coagulation process, and the clotting time is then measured in seconds. In this method 25µl plasma was added into cuvette. 25µl aPTT (Contains negatively charged phospholipids i.e., kaolin) was added to plasma and incubated exactly for 5 minutes. Cuvette was transferred to measuring position. 25µl prewarmed calcium chloride was added and the result was displayed in seconds[19].

Ex-vivo clot lysis assay

A white clot was formed in citrated plasma by treating plasma with thrombin, thrombomodulin, and calcium chloride. The time to lysis of this clot due to inhibition of TAFIa by the addition of PTCI was measured at different concentrations of PTCI in prescence of t-PA. Plasma was added into different wells of a microplate (15 µl). In the control well plasma was treated with HEPES buffer, thrombomodulin (10 µl), t-PA (6.5 µl) and thrombin-Calcium chloride solution (36 µl). Test plasma samples contained all of the above alongwith different doses of PTCI. The total volume in the well was fixed at 120 µl. The buffer volume was changed according to the increasing volume of PTCI stock solution to maintain well volume at 120 µl. Absorbance was measured for 120 mins at 405 nm.[20]

Statistical Analysis

Results were expressed as mean ± SEM. Effect of PTCI on TTO, PT, aPTT, and bleeding time were compared using a one-way analysis of variance followed by Dunnett's tests using Graph Pad Prism software.

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

Results

Aspirin exhibited dose dependent effect in ferric chloride induced thrombosis in rats tested at 25 and 50 mg/kg. Aspirin significantly increased the occlusion time when compared to control group (Fig: 2). In similar pattern to aspirin , Clopidogrel shows dose dependent effect in ferric chloride induced thrombosis at 3, 5 and 10 mg/kg indicated by significant increase in occlusion time as compared to control (Fig:3). While PTCI shows significant effect at 4 mg/kg comparative to control (Fig;4).

In bleeding time experiment , aspirin (200mg/kg) and clopidogrel (10 mg/kg) exhibited significant increase in bleeding time while PTCI failed to reach the level of significance when compared to control (Fig:5). In aPTT assay, all the tested drugs did not showed any significant effect (Fig: 6). In clot lysis assay, PTCI shows significant effect at 0.83 µg/mL comaparative to control (Fig: 7)

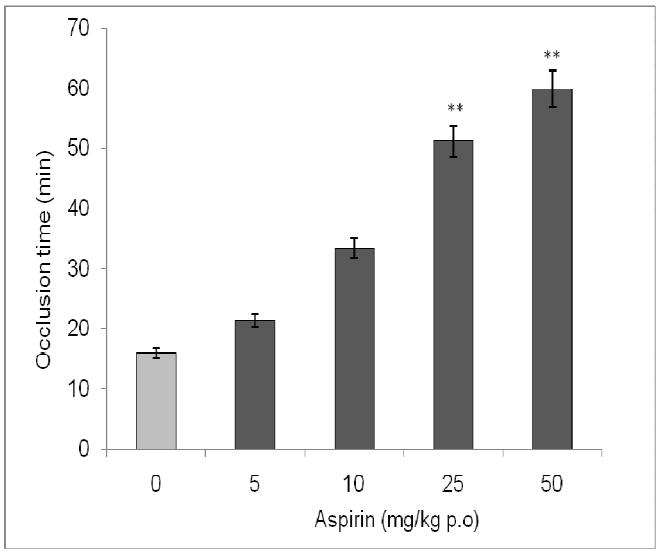

Figure 2

Effect of Aspirin in $FeCL_3$ induced. The column bar represent mean occlusion time .**p<0.01 vs control. n=6

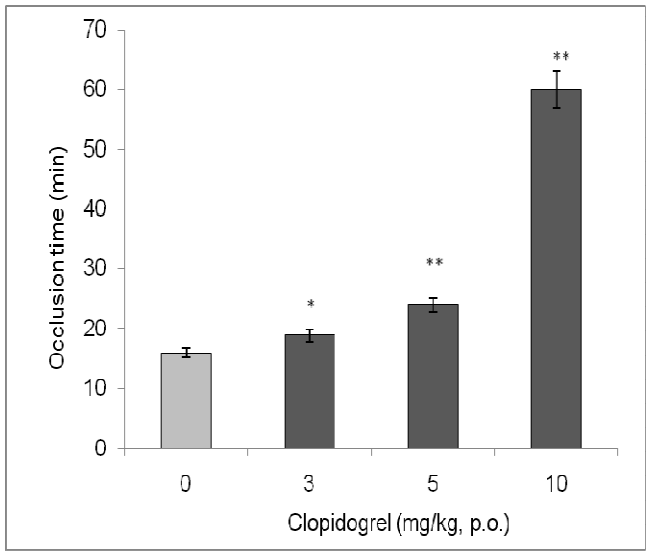

Figure 3

Effect of Clopidogrel in $FeCL_3$ induced model. The column bar represent mean occlusion time .**p<0.01 vs control, *p<0.05 vs control. n=6

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

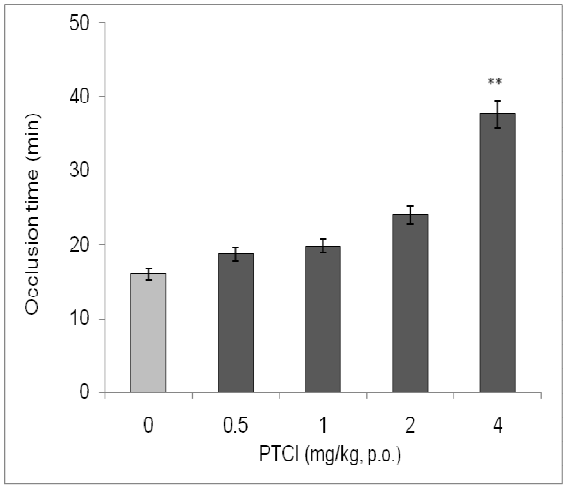

Figure 4

Effect of PTCI in $FeCL_3$ induced model. The column bar represent mean occlusion time .**p<0.01 vs control . n=6

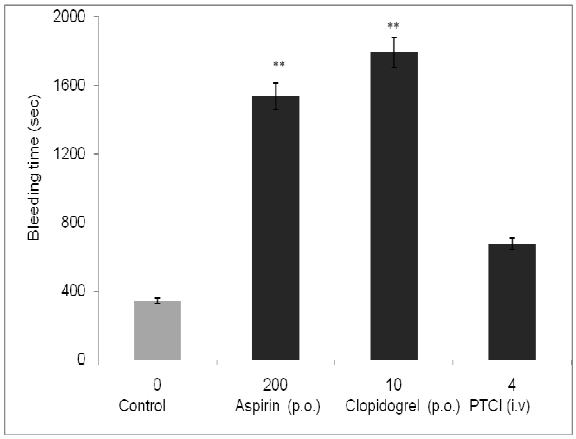

Figure 5

Effect of Aspirin, Clopidogreel and PTCI in bleeding time model model. Each bar graph shows mean+_ sem.** shows p<0.01 from control.

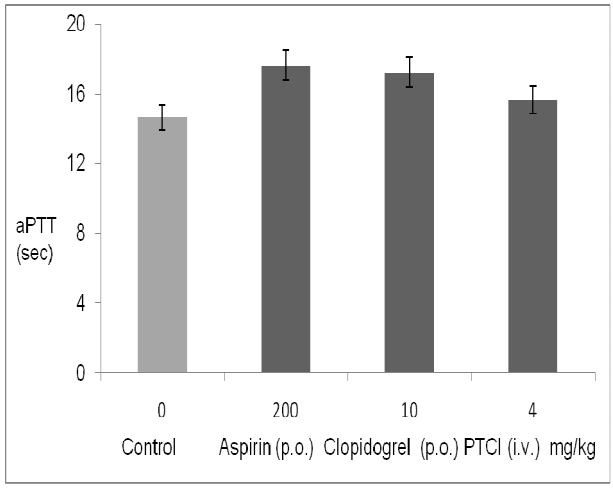

Figure 6

Effect of Aspirin, Clopidogreel and PTCI in activated partial thromboplastin model model.

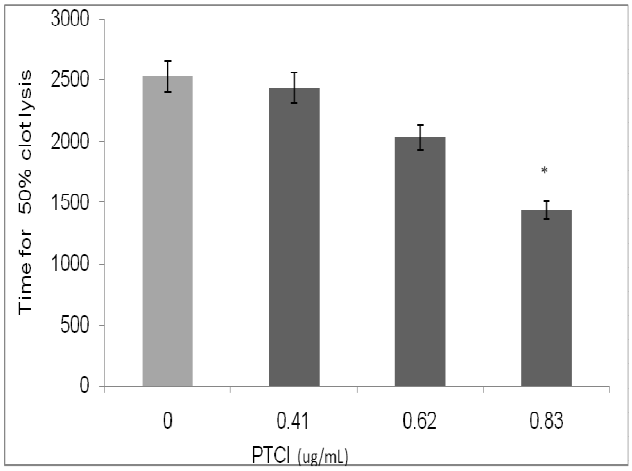

Figure 7

Effect of Aspirin, Clopidogreel and PTCI in bleeding time model model. Each bar graph shows mean ± sem.* shows p<0.01 from control.

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

Discussion

In the current study, PTCI shows significant and dose dependent effect in ferric chloride induced thrombosis model and Ex-vivo clot lysis assay which is as per the hypothesis because it affects fibinolytic cascade alone. While in bleeing time, aPTT studies it shows no significant effect with respect to control, also as per the hypothesis because these models involve only coagulation pathway.

In $FeCl_3$ induce thrombosis model 35% $FeCl_3$ for 5 min is used because at lower concentration (32%) it shows no consistent effect and at 30 % there is no clot formatin at all. In this model aspirin, clopidogrel and PTCI shows dose dependent response, Aspirin and Clopidogrel prove to be very effective drug. These drugs inhibit formation of clot while PTCI act by lysis of clot.

In bleeding time model PTCI not shows any significant effect on this model because there is no or very less role of fibrinolytic pathway in this model. In the determination of aPTT not any drug shows significant increase in aPTT

Conclusion

TAFI has multiple biological effects that include platelet activation, proinflammatory mediation, leukocyte modulation, prothrombotic effect, and endothelial modulation so TAFI can be a important target for various thrombotic disorders, diabetes, obesity and other heart related disorders. Its level can be decrease by PTCI significantly comparative to control.

Effect of PTCI can be increase by using intravenous infusion of t-PA. furher studies are going on to evaluate the inhibitors of TAFI in models like in-vitro clot lysis assay. Various companies are working on different TAFI inhibitors and some major players in this field such as AstraZeneca, Bayer Schering Pharma, Boehringer Ingelheim, Daiichi Sankyo, Pfizer Sanofi-Aventis, Wyeth.

Acknowledgement

Author thankful to Dr. Mukul R. Jain and Dr. Ajay Sharma, Pharmacology Department, Zydus Research Centre for providing apportunity to work and necessary resources for this study.

References

1. Hendriks D., Scharpe S., Van sande, M. Characterization of a carboxypeptidase in human serum distinct from carboxypeptidase N, J. Clin. Chem. Clin. Biochem 27:277–285 (1989).
2. Campbell W, Okada H. An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits, Biochem. Biophys. Res Commun 162: 933–939 (1989).
3. Eaton DL, Malloy BE, Tsai SP. Isolation, molecular cloning, and partial characterization of a novel Carboxypeptidase B from human plasma, J Biol Chem 266 (32): 21833–21838 (1991)

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models

4. Dundar Y, Hill R, Dickson R. Comparative efficacy of thrombolytics in acute myocardial infarction: a systematic review Q J Med 96:103–113 (2003) .
5. Gore JM,Granger CB, Simoons ML. Stroke after thrombolysis: mortality and functional outcomes in the GUSTO-I trial. Circulation 92: 2811-2818 (1995).
6. Meigs JB, D'agostino RB, Wilson PWF. Risk variable clustering in the insulin resistance syndrome. Diabetes 46:1594–1600 (1997).
7. Guven GS, Kilicaslan A, Oz SG. Decrements in the Thrombin Activatable Fibrinolysis Inhibitor (TAFI) Levels in Association with Orlistat Treatment in Obesity Clinical and Applied Thrombosis/Hemostasis, 12 (3) 364-368 (2006)
8. Hass JM, Ryan CA. Methods Enzymol. 80**,** 778–791.
9. Ryan CA. Purification and properties of a carboxypeptidase inhibitor from potatoes. J Biol Chem 249: 5495-5499 (1974).
10. Anonymus. A Randomized, Blind Trial of Clopidogrel Versus Aspirin In Patients At Risk of Ischemic Events (CAPRIE). CAPRIE steering Committee, Lancet 348:1329-1339 (1996).
11. Coukell A and Markham A. Clopidogrel. Drugs 54:745-750 (1997).
12. Gachet C Platelet activation by ADP: the role of ADP antagonists. Ann.Med 32 Suppl 1:15-20.:15-20 (2000).
13. Hechler B, Eckly A, Ohlmann P, Cazenave JP, and Gahet C. The p2y1 receptor, necessary but not sufficient to support full ADP induced platelet aggregation, is not the target of the drug Clopidogrel. Br.J Haematol. 103:858-866 (1998).Bhawan J, Joris I, Girolami U, and Majno J Effect of cclusion on large vessels, I.A study of the rat carotid cartery, Am.J Pathol. 88:355-380 (1977).
14. Kurz KD, Main BW, and Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. Thromb.Res. 60:269-280 (1990).
15. Wang YX, Dong N, Wu C, Martin-McNulty B, Fitch RM, Vicelette J, Tran K, Vergona R, Sullivan ME, Morser J, and Wu Q. Lipopolysaccharide attenuates thrombolysis in batroxobin-induced lung vasculature fibrin deposition but not in ferrous chloride-induced carotid artery thrombus in rats: role of endogenous PAI-1. Thromb.Res. 111:381-387 (2003).
16. Herbert JM, Bernat A, Maffrand JP. Aprotinin reduces clopidogrel-induced prolongation of the bleeding time in the rat. Thromb.Res. 71:433-441 (1993).
17. Oldgren J, Linder R, Grip L, Siegbahn A, Wallentin L. Activated partial thromboplastin time and clinical outcome after thrombin inhibition in unstable coronary artery disease. Eur Heart J. 20(22):1657-66 (1999)
18. Kurata M, Noguchi N, Kasuga Y, Sugimoto T, Tanaka K, Hasegawa T. Prolongation of PT and APTT under excessive anticoagulant in plasma from rats and dogs. J Toxicol Sci. 23(2):149-53 (1998).
19. Nagashima M, Yin ZF, Zhao L, White K, Zhu Y, Lasky N, Halks-Miller M, Broze GJ Jr, Fay WP, Morser J. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. J Clin Invest; 109: 101–110 (2002).

Evaluation of potato tuber carboxypeptidase inhibitor with standard anti-thrombotic drugs in various thrombosis models