IMPROVING THE UNDERSTANDING OF PLASMA KALLIKREIN CONTRIBUTION TO ARTERIAL THROMBUS FORMATION USING TWO PLANT PROTEASE INHIBITORS

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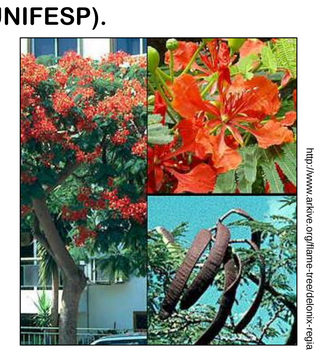
SUMMARY

Introduction

The purpose of antithrombotic therapy is the prevention of thrombus formation and/or its extension with a minimum risk of bleeding. The inhibition of a variety of proteolytic processes, especially those of the coagulation cascade, has been reported as a property of plant protease inhibitors. The role of trypsin inhibitors (TIs) from *Delonix regia* (Dr) and *Acacia schweinfurthii* (As), members of the Kunitz family of protease inhibitors, was investigated on blood coagulation platelet aggregation and thrombus formation. This study was authorized by the ethics committee, CEP 0193/06 (UNIFESP).



http://www.arkive.org/flame-tree/delonix-regia

Delonix regia

http://www.zimbabweflora.co.zw

Acacia schweinfurthii

Objective

In the present study, we evaluated the importance of plasma kallikrein in *in vitro* coagulation assays, platelet aggregation induced by ADP and arterial thrombosis using two plant protease inhibitors.

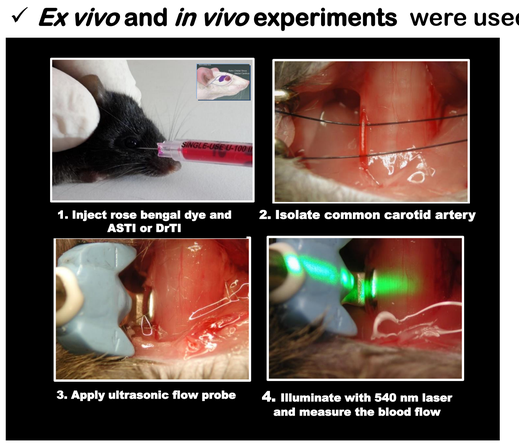
MATERIAL and METHODS

✓ **Inhibitors and kinetic studies:** the residual activity of huPK was measured by the hydrolysis of 0.8 mM HD-Pro-Phe-Arg-pNan in 0.1 M Tris-HCl buffer, pH 8.0, at 37ºC, The hydrolysis of HD-Pro-Phe-Arg-pNan was monitored by measuring the absorbance of released p-nitroaniline at 405 nm in a Spectra MAX plus 384 spectrophotometer (Molecular Devices). FXIa activity was measured by the hydrolysis of 0.2 mM Boc-Glu(Obzl)-Ala-Arg-AMC.HCl in 20 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 5 mM CaCl2, and 0.1% BSA at 37oC.. The fluorescence of Boc-Glu(Obzl)-Ala-Arg-AMC.HCl was measured at 380/460 nm in a Spectra Gemini EM (Molecular Devices). The Kiapp values were estimated in triplicate by measuring the effect of increasing concentrations of the inhibitor on enzyme activity using non-linear regression analysis in the Grafit program version 4.0 (Erithacus Software, Staines, UK) for slow-tight binding.

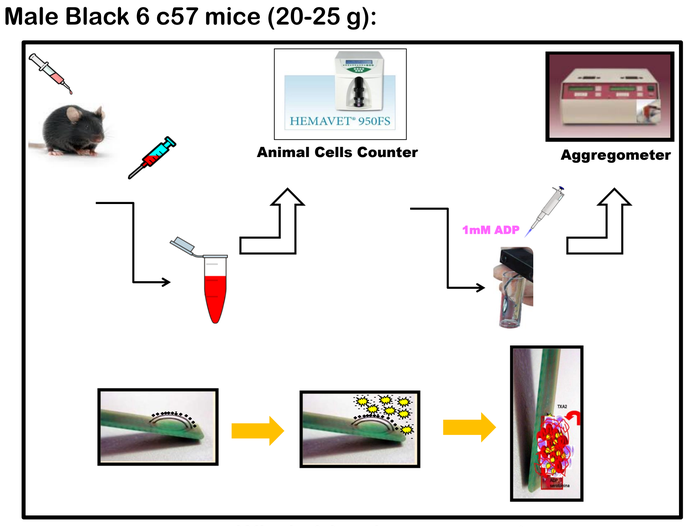
✓ ***In vitro*** **coagulation assays: Prothrombin Time** (PT) and **Partial Thromboplastin Time (aPTT)** were determined in human plasma using a semi-automated BFT II coagulometer (Dade Behring);

✓ **Statistical comparisons** among groups were performed using ANOVA and *p < 0.05, **p < 0.01, and ***p<0.001 was considered significant (GraphPad InStat).

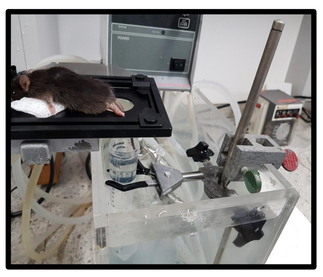
✓ ***Ex vivo*** **and** ***in vivo*** **experiments** were used Male Black 6 c57 mice (20-25 g):



Photochemical model arterial thrombosis model



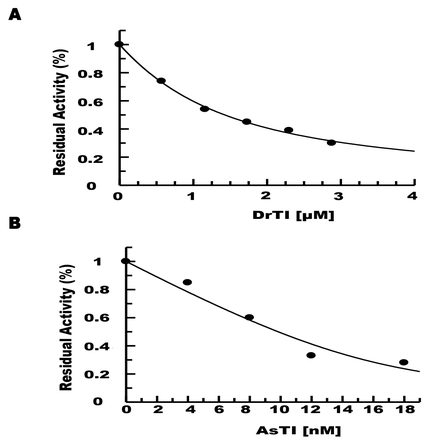
Whole blood aggregometry



Bleeding time

RESULTS

Effects of inhibitors on proteolytic enzymes



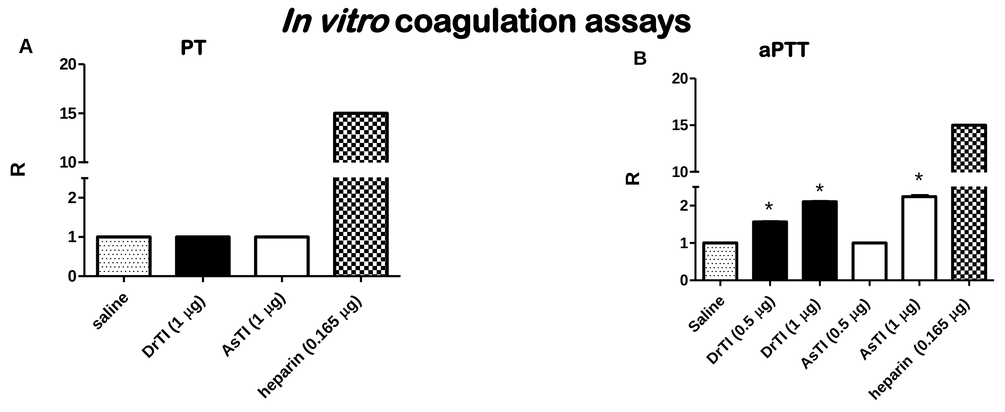
C

Enzymes	Inhibitor (K_{iapp}, M)	
	DrTI	AsTI
huPK	5.25×10^{-9}	1.6×10^{-9}
Trypsin	2.2×10^{-8}	3.45×10^{-9}
FXa	N.I.	N.I
FXIa	1.3×10^{-6}	N.I

N.I – No Inhibition

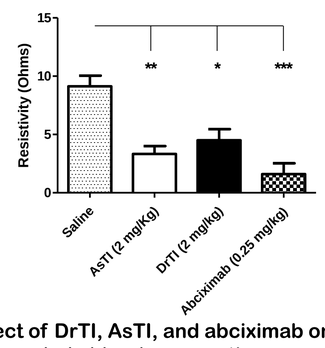
Determination of Kiapp values of (A) DrTI for FXIa inhibition, and (B) AsTI for huPK inhibition. The graphs show the percentage of residual activities of enzymes as a function of the inhibitor's concentration. (C) Kiapp for different enzymes.

In vitro coagulation assays



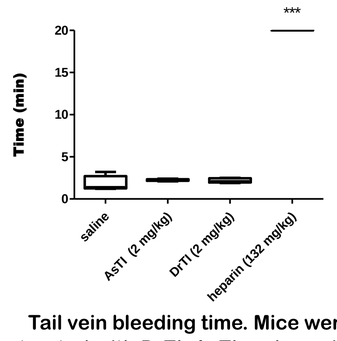
Effect of DrTI (black) and AsTI (white) on the following hemostatic parameters: PT prothrombin time (A), aPTT activated partial thromboplastin time (B).

Whole blood aggregometry



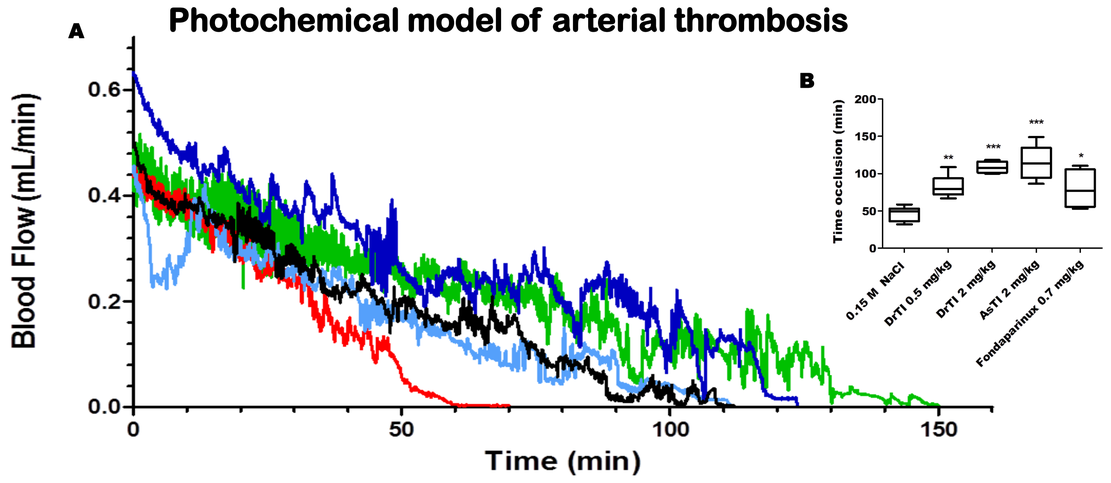
Effect of DrTI, AsTI, and abciximab on whole blood aggregation.

Bleeding time



Tail vein bleeding time. Mice were treated with DrTI, AsTI, or heparin.

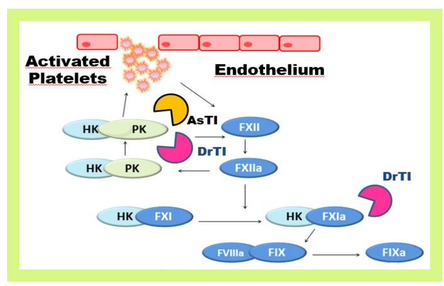
Photochemical model of arterial thrombosis

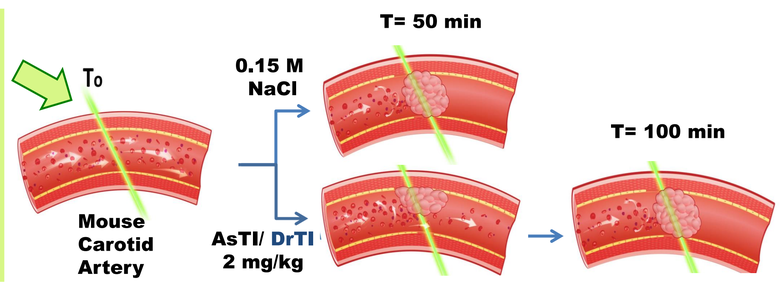


(A) Carotid artery blood flow curves in C57 Black 6 mice. Comparison between NaCl (0.15 M in 11 mice in red), fondaparinux (0.7 mg/kg in 7 mice in black), DrTI (0.5 mg/kg in 6 mice in light blue), DrTI (2 mg/kg in 5 mice in dark blue), and AsTI (2 mg/kg in 5 mice in green). **(B)** Measurement of time to show thrombotic vascular occlusion in C57 Black 6 mice.

CONCLUSIONS

- Plant protease inhibitors, DrTI and AsTI, decreased thrombus formation without affecting the bleeding time, demonstrating the inhibition of PK can be regarded as a strategy for the treatment and prevention of thrombosis and are promising targets for the development of new anticoagulant drugs.





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Animal Models in Thrombosis & Hemostasis

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