Inhibition of the amplification reactions of blood coagulation by site-specific inhibitors of $\alpha$-thrombin

Frederick A. OFOSU,*†† John W. FENTON, II,‡ John MARAGANORE,§ Morris A. BLAJCHMAN,*†† Xianjun YANG,† Lindsay SMITH,* Noorildan ANVARI,† Michael R. BUCHANAN† and Jack HIRSH||
* The Canadian Red Cross Society, Blood Transfusion Service, Hamilton, Ont. L8N 1H8, Canada,
† Department of Pathology, McMaster University, Hamilton, Ont. L8N 3Z5, Canada,
|| Department of Medicine, McMaster University, Hamilton, Ont. L8N 3Z5, Canada

Hirudin and hirulog-1 (D-Phe-Pro-Arg-Pro-[Gly]$\alpha$-desulphohirudin-(54–65)) abrogate the enzyme activities of $\alpha$-thrombin by binding the enzyme simultaneously at its catalytic centre and fibrinogen-recognition exosite. In contrast, hirugen [hirudin-(54–65)] binds $\alpha$-thrombin solely at the fibrinogen-recognition exosite, and competitively inhibits fibrinopeptide A release. To investigate the extent to which the fibrinogen-recognition exosite is involved when $\alpha$-thrombin catalyses the amplification reactions of coagulation, we compared the abilities of hirudin, hirulog-1 and hirugen to inhibit simultaneously Factor X, Factor V and prothrombin activation. Whereas 0.1 $\mu$M-hirudin and 0.1 $\mu$M-hirulog-1 (i.e. less than 10% of the concentration of prothrombin in plasma) inhibited Factor X, Factor V and prothrombin activation, 10 $\mu$M was the minimum concentration of hirugen to achieve a similar anticoagulant action. Concentrations of hirudin and hirulog-1 equimolar to 5 times greater than those of $\alpha$-thrombin respectively abrogated Factor V activation by exogenous $\alpha$-thrombin. In contrast, a 500-fold molar excess of hirugan could not. The inability of hirugen to inhibit the activation of the three clotting factors effectively suggests that the fibrinogen-recognition exosite does not play a mandatory role when thrombin activates Factor V.

INTRODUCTION

$\alpha$-Thrombin accelerates its own generation by activating Factor V and Factor VIII. For this reason, agents able to effect the inhibition of thrombin suppress prothrombin activation in plasma. For example, submicromolar concentrations of hirudin and heparin inhibit prothrombin activation by inhibiting the activation of Factor VIII and Factor V normally catalysed by endogenous $\alpha$-thrombin [1–4]. $\alpha$-Thrombin consists of a smaller A chain linked by a disulphide bond to the B chain [5]. The B chain has the catalytic triad and the fibrinogen-recognition exosite important for the high specificity of $\alpha$-thrombin towards fibrinogen [6–8]. Hirudin abrogates all enzymic functions of $\alpha$-thrombin by forming a stable ($K_i$ approx. 10 $\mu$M) but non-covalent complex with the enzyme [9–12]. The three-dimensional structures of r-hirudin variant 2-Lys-47 and r-hirudin variant 1 complexed to $\alpha$-thrombin demonstrate that the apolar compact N-terminal head domain of hirudin binds $\alpha$-thrombin in a manner that blocks access to the active-site cleft of the enzyme [13,14]. The C-terminal tail domain of hirudin occupies the fibrinogen exosite, which is adjacent to the catalytic cleft of the enzyme, where it makes numerous electrostatic and hydrophobic contacts with the exosite [13,14].

Complexing of hirudin to $\alpha$-thrombin is postulated to begin at the C-terminal tail of the inhibitor [13], and tail fragments of hirudin competitively inhibit the clotting of fibrinogen by $\alpha$-thrombin [15–19]. Thus, with respect to fibrinogen, hirugen transiently converts $\alpha$-thrombin into a $\gamma$-thrombin-like enzyme. The fibrinogen-recognition exosite is essentially deleted in $\gamma$-thrombin, and this enzyme has less than 0.5% of the clotting activity of $\alpha$-thrombin [8]. Hirulog-1 is a bivalent derivative of hirudin with the structure D-Phe-Pro-Arg-Pro-[Gly]$\alpha$-desulphohirudin-(54–65) [16]. It combines the high affinity of D-Phe-Pro-Arg-CH$_2$Cl for the catalytic centre of $\alpha$-thrombin [20] with the affinity of hirudin-(54–65) for the fibrinogen-recognition exosite [16–18]. Hirulog-1 inhibits fibrinopeptide A release and the amidolytic activity of thrombin with $K_i$ of 144 and approx. 2 nM respectively [15,16], whereas natural and recombinant hirudins inhibit fibrinopeptide A release and the amidolytic activity of thrombin with $K_i$ in the femtomolar to picomolar range [9–12]. Although hirudin and hirulog-1 effect a dose-dependent prolongation of the activated partial thromboplastin time, prolongation of this test by hirugan is saturable [16,18,19]. During the activated partial thromboplastin time test, prothrombin is converted into $\alpha$-thrombin, which then clots fibrinogen. The saturable prolongation of this test by hirugan raises the possibility that, unlike hirudin [4] and hirulog-1, hirugan cannot significantly inhibit prothrombin activation. The present study investigated the validity of this hypothesis by determining the extents to which hirudin, hirulog-1 and hirugan could inhibit Factor X, Factor V and prothrombin activation. Factor X and Factor V activation are requisite events for efficient prothrombin activation in plasma.

MATERIALS AND METHODS

Materials

Recombinant hirudin was a gift from Dr. R. B. Wallis, CIBA-GEIGY, Horsham, Sussex, U.K. Hirugan and hirulog-1 were synthesized and characterized by using previously described procedures [12,14]. Purified human Factor V was obtained from Haematologic Technologies, Essex Junction, VT, U.S.A., and labelled with Na$^{125}$I to a specific radioactivity of 20 $\mu$Ci/mg, by procedures previously employed to iodinate prothrombin and Factor V [17,19]. Rabbit anti-(human prothrombin) serum used as the second antibody in the e.l.i.s.a. to quantify prothrombin.

Abbreviation used: APTT, activated partial thromboplastin time.

* To whom correspondence should be addressed, at the Department of Pathology, McMaster University.
consumption [20] was obtained from Behring Diagnostics, Montreal, Quebec, Canada. d-Phe-Pro-Arg-CH₂Cl and dansyl-Glu-Gly-Arg-CH₂Cl, chloromethane inhibitors of α-thrombin and Factor Xa respectively [20,21], were obtained from Terachem, Toronto, Ontario, Canada. A mouse monoclonal antibody to Factor X was obtained from American Diagnostica, Greenwich, CT, U.S.A. Reagents and materials for PAGE were obtained from Bio-Rad Laboratories, Mississauga, Ontario, Canada. Activated partial thromboplastin time (APTT) reagent was obtained from Organon Technika, Toronto, Ontario, Canada. Rabbit brain tissue factor was a gift from Dr. Leon Polier, National Centre for Anticoagulant Reagents and Control, Manchester, U.K. Fatty acid-free BSA, other reagents and chemicals were obtained from Sigma Chemical Co., Mississauga, Ontario, Canada.

**Prothrombin and Factor X activation and their inhibition by hirudin, hirulog-1 and hirugen**

Activation of prothrombin in defibrinated plasma was initiated either by contact activation (intrinsic activation) or by rabbit brain tissue factor (extrinsic activation). Contact activation was effected by adding 1 vol. of APTT reagent to 2 vol. of plasma. After a 5 min incubation at 37 °C, 1 vol. of 40 mM-CaCl₂ (prewarmed to 37 °C) was added to initiate intrinsic coagulation. Samples were withdrawn at 15 s intervals into 4 vol. of a buffer containing 145 mM-NaCl, 36 mM-sodium barbiturate, 36 mM-sodium acetate, 10 mM-EDTA, 1 μM recombinant hirudin, 5 units of heparin/ml and 0.1 mg of fatty acid-free BSA/ml at pH 7.4. This buffer served three functions. By chelating Ca²⁺, the EDTA stopped further Factor X and prothrombin activation. Hirudin inactivated any free thrombin, thus eliminating competition between thrombin and Factor Xa for antithrombin III. Heparin accelerated the inhibition of Factor Xa by antithrombin III [22]. After a 10 min incubation at 37 °C, the samples were frozen at −50 °C until the concentrations of prothrombin consumed and total Factor-Xa-antithrombin-III generated were assayed. When Factor X and prothrombin activation were initiated by adding tissue factor and CaCl₂ to plasma, undiluted rabbit brain tissue factor replaced the APTT reagent. The e.l.i.s.a.s used to quantify prothrombin consumption and total Factor Xa-antithrombin III have been described previously [4,22]. However, the first antibody used in this study to bind Factor Xa-antithrombin III to the micro-titre plates was a monoclonal antibody to Factor X. When coated to micro-titre plates at a concentration of 300 ng/ml, this antibody was found to react equally well with Factor X, Factor Xa and Factor Xa-antithrombin III. The effects of hirudin (0.1 μM and 1 μM), hirulog-1 (0.1 μM, 1 μM and 10 μM) and hirugen (0.1 μM, 1 μM and 10 μM) on prothrombin and Factor X activation were also investigated.

**Factor V activation and its inhibition by hirudin, hirulog-1 and hirugen**

Proteolysis of ¹²⁵I-labelled Factor V resulting from CaCl₂ addition to contact-activated plasma, and that resulting from tissue factor and CaCl₂ addition to plasma, were determined. ¹²⁵I-labelled Factor V was added to plasma to a final concentration of 10 ng/ml. Samples of the recalcified plasmas were added to equal volumes of 5 mM-EDTA, 1 μM-d-Phe-Pro-Arg-CH₂Cl and 1 μM-dansyl-Glu-Gly-Arg-CH₂Cl in 36 mM-sodium barbiturate/36 mM-sodium acetate buffer, pH 7.4, to stop further Factor V proteolysis. Electrophoretic buffer (80 μl) was then added to 20 μl of each sample, and a 50 μl sample was subjected to electrophoresis in 5–15% gradient polyacrylamide gels containing 0.1% SDS [16]. The gels were dried under vacuum and ¹²⁵I-labelled Factor V and its proteolytic derivatives were detected by exposing the gels to X-ray films for up to 7 days [16].

**RESULTS**

**Inhibition of Factor X activation by hirudin, hirulog-1 and hirugen**

A coagulant surface, Factor Xa and Factor Va are required for optimizing prothrombin activation. We therefore quantified Factor X activation and the effects of hirudin, hirulog-1 and hirugen on Factor X activation. As none of the three inhibitors of α-thrombin inactivates Factor Xa, suppression of Factor Xa-antithrombin III formation by each agent directly reflected the extent to which it could inhibit Factor X activation. Approx. 1 nM-Factor Xa was generated 30 s after CaCl₂ was added again to control contact-activated plasma (Fig. 1), and this value had increased to 185 nm 60 s later (Fig. 1). A minimum incubation of 75 s and 90 s were required before Factor X activation could be

![Fig. 1. Inhibition of intrinsic Factor X activation by hirudin (a) hirulog-1 (b) and hirugen (c)](image)

Intrinsic Factor X activation was initiated by adding CaCl₂ to contact-activated plasma. Free Factor Xa in each sample was converted into Factor Xa-antithrombin III by adding 5 units of heparin/ml. The concentration of total Factor Xa-antithrombin III in each sample (i.e. endogenous Factor Xa-antithrombin III plus free Factor Xa) was measured by an e.l.i.s.a. for Factor Xa-antithrombin III. Control contact-activated plasma; ○, 0.1 μM-inhibitor; □, 1.0 μM-inhibitor; ■, 10 μM-inhibitor.

![Fig. 2. Inhibition of extrinsic Factor X activation by hirudin (a) hirulog-1 (b) and hirugen (c)](image)

Extrinsic Factor X activation was initiated by adding rabbit brain tissue factor and CaCl₂ to plasma. Free Factor Xa in each sample was converted into Factor Xa-antithrombin III by adding heparin (5 units/ml) to each sample. The concentration of total Factor Xa-antithrombin III was measured by e.l.i.s.a. Control plasma; ○, 0.1 μM-inhibitor; □, 1.0 μM-inhibitor; ■, 10 μM-inhibitor.
Anticoagulant mechanisms of r-hirudin and truncated hirudins

Intrinsic pathway activation of $^{125}$I-labelled Factor V was initiated by adding CaCl$_2$ to contact-activated plasma. After various incubation times, the resulting proteolysis of $^{125}$I-labelled Factor V was estimated by SDS/PAGE and autoradiography. (a) The recalification times were as follows: lane 1, 0 s; lane 3, 30 s; lane 4, 45 s; lane 5, 60 s. $M_r$ markers were applied in lane 2. (b) Plasma containing 1 $\mu$m-hirudin was recalified as follows: lane 1, 45 s; lane 2, 60 s; lane 3, 90 s. (c) Plasma containing 1 $\mu$m-hirulog-1 was recalified as follows: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s; lane 4, 0 s. $M_r$ markers were applied in lane 5. (d) Plasma containing 10 $\mu$m-hirulog was recalified for the following incubation times: lane 1, 45 s; lane 2, 60 s; lane 3. 90 s. Key: V, Factor V; A, Factor V activation peptide; H and L represent the Factor Va heavy and light chains respectively.

Intrinsic activation of Factor V in control plasma (a), and in plasma containing 1.0 $\mu$m-hirulog-1 (c) and 10 $\mu$m-hirulog (d)

Intrinsic pathway activation of $^{125}$I-labelled Factor V was initiated by adding CaCl$_2$ to contact-activated plasma. After various incubation times, the resulting proteolysis of $^{125}$I-labelled Factor V was estimated by SDS/PAGE and autoradiography. (a) The recalification times were as follows: lane 1, 0 s; lane 3, 30 s; lane 4, 45 s; lane 5, 60 s. $M_r$ markers were applied in lane 2. (b) Plasma containing 1 $\mu$m-hirulog was recalified as follows: lane 1, 45 s; lane 2, 60 s; lane 3, 90 s. (c) Plasma containing 1 $\mu$m-hirulog-1 was recalified as follows: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s; lane 4, 0 s. $M_r$ markers were applied in lane 5. (d) Plasma containing 10 $\mu$m-hirulog was recalified for the following incubation times: lane 1, 45 s; lane 2, 60 s; lane 3. 90 s. Key: V, Factor V; A, Factor V activation peptide; H and L represent the Factor Va heavy and light chains respectively.

A suspension of rabbit brain tissue factor and CaCl$_2$ (at 37°C) was added to plasma preincubated to 37°C. After various incubation times at 37°C, the proteolysis of $^{125}$I-labelled Factor V was assessed by SDS/PAGE and autoradiography. (a) The recalification times of control plasma were as follows: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s; lane 4, 0 s. Radioactive $M_r$ markers were applied in lane 5. (b) Plasma containing 1 $\mu$m-hirudin was recalified as follows: lane 1, 45 s; lane 2, 90 s; lane 3, 135 s. (c) Plasma containing 1 $\mu$m-hirulog-1 was recalified as follows: lane 1, 0 s; lane 3, 45 s; lane 4, 90 s; lane 5, 135 s. $M_r$ markers were applied in lane 2. (d) Plasma containing 10 $\mu$m-hirulog was recalified as follows: lane 1, 45 s; lane 2, 60 s; lane 3, 90 s. V, A, L and H are as defined in Fig. 3 legend.

10 $\mu$m-hirulog inhibited extrinsic Factor X activation as effectively as 0.1 $\mu$m-hirudin or 0.1 $\mu$m-hirulog-1 (Fig. 2c).

Intrinsic Factor V activation: inhibition by hirudin, hirulog-1 and hirulog

Before the addition of CaCl$_2$ to plasma, $^{125}$I-labelled Factor V (approx. $M_r$ 330000) and several derivatives (all with apparent $M_r$ > 130000) were detected in plasma (Figs. 3 and 4). Proteolysis of $^{125}$I-labelled Factor V resulting in production of Factor Va heavy (H) and light (L) chains and Factor V activation peptide (A) became evident 30 s after CaCl$_2$ was added to contact-activated plasma (Fig. 3a, lane 3). Subsequently, the largest Factor V fragment detectable was the activation peptide, $M_r$ approx. 150000 (Fig. 3a, lane 4 and 5). Hirudin (1 $\mu$m) delayed the onset of intrinsic Factor V activation and proteolysis of Factor V had not begun (Fig. 3b, lanes 1-3). At 1.0 $\mu$m, hirulog-
The minimum concentration of hirudin required to cleave plasma Factor V to the same extent as 20 nM-
α-thrombin (results not shown).}

**Inhibition of prothrombin activation by hirudin, hirulog-1 and hirugen**

Prothrombin activation began 30 s after CaCl₂ was added to control contact-activated plasma, and was essentially complete 60 s later (Fig. 5). The presence of 0.1 μM and 1.0 μM-hirudin delayed the onset of prothrombin consumption by 30 s and 60 s respectively (Fig. 5a). As summarized in Fig. 5(b), 1.0 μM-hirulog-1 delayed the onset of intrinsic prothrombin activation by 30 s, and thus was as effective (Fig. 5d) as 0.1 μM-hirudin. Hirugen was less effective than hirudin or hirulog-1, as 10 μM-hirugen could delay the onset of prothrombin activation by only 15 s (Fig. 5c). Prothrombin consumption by the extrinsic pathway was complete 60 s after its initiation (Fig. 6). Hirudin inhibited extrinsic prothrombin activation less effectively than it could inhibit intrinsic prothrombin activation (Fig. 6a). The minimum concentrations of hirulog-1 and hirugen able to delay the onset of extrinsic prothrombin activation by more than 10 s were 1.0 μM and 10 μM respectively (Figs. 6b and 6c).

**DISCUSSION**

The antithrombins D-Phe-Pro-Arg-CH₂Cl and hirudin delay the onset of prothrombin activation by inhibiting the amplification reactions of coagulation normally catalysed by α-thrombin generated in situ[1,3,4]. D-Phe-Pro-Arg-CH₂Cl inactivates thrombin by alkylating the histidine member of the catalytic triad of the enzyme[20,23]. Recombinant hirudins inactivate α-thrombin by forming a tight but non-covalent complex with the enzyme. The N-terminal apolar head domain of hirudin blocks access to the catalytic centre, whereas the anionic tail of hirudin binds the enzyme at the exosite required for fibrinogen recognition[13,14]. Beginning with hirudin-(54-65) (hirugen), tail fragments of hirudin bind α-thrombin at the fibrinogen-recognition exosite and act as competitive inhibitors of fibrinopeptide A release[15-19].

The present study determined whether the fibrinogen-recognition exosite is essential when α-thrombin activates Factor V. We found that 20 nM-exogenous α-thrombin activated Factor V in plasma within 15-30 s. Concentrations of hirudin inhibi
Anticoagulant mechanisms of r-hirudin and truncated hirudins

Pro-[Gly]4-desulphohirudin-(54–65) [16]. The first three residues have a high affinity for the catalytic site of α-thrombin [20, 23–25]. The fourth residue, proline, was added to retard cleavage of the affinity ligand by α-thrombin [26]. The [Gly]4 spacer arm provides the minimum distance to optimize the simultaneous binding of hirulog-1 to the enzyme at the catalytic centre and the fibrin(ogen)-recognition exosite [27]. On the basis of its binding affinity, the affinity ligand is consistent with the saturable effects that activation of Factor VIII has for the catalytic centre and the fibrinogen exosite [27]. The affinity ligand with the saturable interaction also consistent with the catalytic centre and the fibrinogen exosite [27]. The affinity ligand with the saturable interaction also consistent with the catalytic centre and the fibrinogen exosite [27].

Either 0.1 μM-r-hirudin or 0.1 μM-hirulog-1 delayed the onset of Factor V and Factor X activation by about 30 s with 10 μM-hirugen required to achieve a similar effect. The reason why the effect of 10 μM-hirugen on extrinsic activation of Factor X is more marked than its effect on the intrinsic activation of this zymogen is not known. The inability of hirugen to inhibit Factor X activation as effectively as hirudin and hirulog suggests that the fibrin(ogen)-recognition exosite of α-thrombin is not crucial for thrombin to activate Factor VIII. Consistent with their effects on Factor V and Factor X activation, all three concentrations of r-hirudin or hirulog-1 inhibited prothrombin activation, whereas 10 μM-hirugen primarily delayed the onset of prothrombin activation. The action of hirugen on prothrombin activation is consistent with the saturable prolongation of both intrinsic and extrinsic coagulation by hirugen [19]. The present data thus indicate that activation of Factor V by α-thrombin to the level required for optimizing prothrombin activation can occur even when the fibrin(ogen)-recognition exosite of α-thrombin is essentially occupied. Nonetheless, effective occupancy of this site (by saturating concentrations of hirugen) does lengthen the time required for saturating concentrations of Factor Va to become available in situ. The fibrin(ogen)-recognition exosite is not necessary when antithrombin III inactivates thrombin [28]. Thus γ-thrombin and α-thrombin are inactivated by antithrombin III at similar rates [29].

There is evidence also consistent with the idea that the site for the initial interactions of Factor V with α-thrombin is not the fibrin(ogen)-recognition exosite. The fifth epidermal growth factor domain of thrombomodulin binds α-thrombin at the fibrin(ogen) exosite [28]. Di-isopropylphosphonated (DIP) α-thrombin bound to cell surface thrombomodulin is displaced by hirudin, hirugen and fibrinogen [28]. In contrast, a synthetic peptide based on a sequence of Factor Va analogous to hirugen cannot displace DIP-α-thrombin from thrombomodulin [28]. These fundamental differences in recognition mechanisms for its various substrates may explain why α-thrombin can activate Factor V at concentrations lower than that required for clotting.

This study was funded in part by Grants-in-Aid from Ontario Heart and Stroke Foundation, Medical Research Council of Canada, and National Institutes of Health Grant HL 13160. The expert assistance of A. Jadeski and M. Hannon-Disko in preparing the typescript is gratefully acknowledged.

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Received 15 April 1991 / 31 October 1991; accepted 11 November 1991