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Inhibition of fluid phase and clot-bound thrombin by the thrombin exosite I ligand LU 58463: comparison with heparin and hirudin

Heparin inhibits clot-bound thrombin less efficiently than fluid phase thrombin. We compared the ability of heparin, a non-selective indirect thrombin inhibitor, r-hirudin, a non-selective bifunctional thrombin inhibitor, and LU 58463, a non-catalytic exosite I thrombin inhibitor, to inhibit fluid-phase and clot-bound thrombin. The thrombin inhibitor LU 58463 was more effective than heparin and as effective as hirudin in inhibiting clot-bound thrombin.

haematologica 2002; 87:1116-1117
 (http://www.haematologica.org/2002_10/1116.htm)

Human α -thrombin has three key functional domains. The catalytic center is the site where substrates are cleaved. The non-catalytic substrate-binding domains, known as anion binding exosite I and II, are involved in fibrin(ogen) and heparin binding, respectively.¹ Thrombin is continuously incorporated into the growing clot by binding to fibrin(ogen) through the anion binding exosite I, so that clot-bound thrombin remains active.² Heparin catalyses thrombin inactivation through the formation of thrombin-antithrombin (AT) complex, while hirudin directly inhibits thrombin by binding to both the catalytic site and the anion binding exosite I. When bound to fibrin, thrombin is resistant to inactivation by the AT-heparin complex but not by hirudin. This difference could explain the improved ability of hirudin over heparin in preventing the extension of venous thrombosis,³ or re-thrombosis after thrombolysis.^{4,5} LU 58463 is a non-catalytic exosite I thrombin inhibitor derived from the C-terminus of hirudin.^{56-65,67}

We compare the ability of heparin, r-hirudin and LU 58463 to inhibit fluid-phase and clot-bound thrombin. In the fluid-phase system 50 μ L of human α -thrombin (final concentration 0.4 nM) were incubated with 450 μ L aliquots of citrated plasma for 60 minutes at 37°C in the presence of increasing concentrations of thrombin inhibitors. In the clot-bound system fibrin clots were formed around siliconized polystyrene coated wire hooks by the addition of CaCl₂ (final concentration 25 mM) to 450 μ L aliquots of fresh citrated plasma. After washing in TBS, fibrin clots were incubated in 1 mL aliquots of fresh citrated plasma for 60 minutes at 37°C in the presence of increasing concentrations of thrombin inhibitors. The generation of FPA was used as an index of thrombin activity.

The three agents were tested over a wide range of concentrations: r-hirudin from 0.1 to 1000 nM; LU 58463 from 0.1 to 2500 nM; unfractionated heparin from 0.025 to 5 anti-Xa U/mL. The ratio of the concentrations of the three inhibitors able to achieve

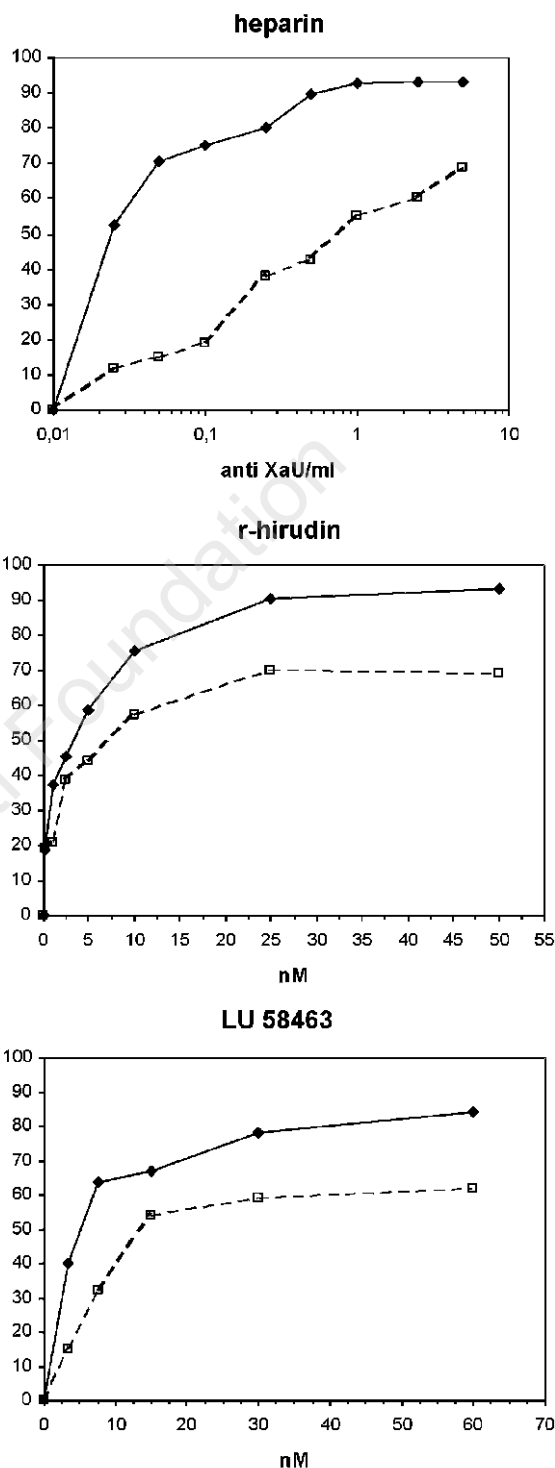


Figure 1. Percentage of inhibition of FPA generation induced by heparin, r-hirudin and LU 58463 in fluid phase and clot-bound thrombin.
 — fluid phase thrombin. - - - clot-bound thrombin.

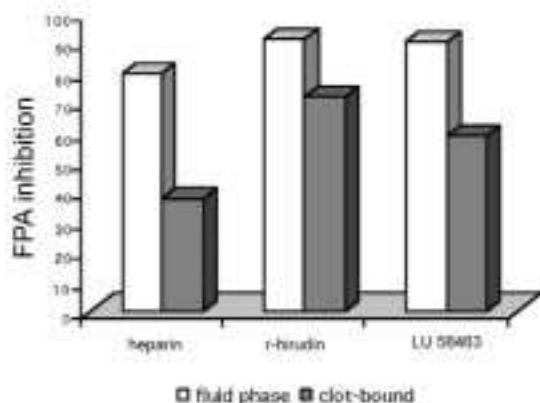


Figure 2. Effect of heparin, r-hirudin and LU 58463 on FPA generation at concentrations doubling the aPTT, in fluid phase and in the clot-bound system.

60% inhibition of FPA generation in the fluid-phase and in the clot-bound systems was identified. The inhibitory effect on fluid-phase thrombin and clot-bound thrombin of the three agents was assessed at concentrations able to double the aPTT.

The ratio of the concentrations able to achieve 60% inhibition of thrombin in the clot-bound and in the fluid phase systems was 20 for heparin, 2 for r-hirudin and 5 for LU 58463 (Figure 1). The concentrations of the inhibitors able to double the aPTT were 0.25 anti-Xa IU/mL for heparin, 100 nM for r-hirudin and 250 nM for LU 58463. These concentrations produced an inhibition of thrombin in fluid phase of 91.0 ± 7.8% (r-hirudin), 89.5 ± 7.2% (LU 58463) and 80.0 ± 18.8% (heparin). At these same concentrations r-hirudin produced an inhibition of clot-bound thrombin of 72.6 ± 18.9% (ratio clot-bound/fluid phase thrombin inhibition = 0.79); LU 58463 of 58.9 ± 9.3% (ratio=0.66) and heparin of 38.2 ± 9.3% (ratio=0.48) (Figure 1). The inhibition ratio of fluid-phase and clot-bound thrombin for heparin was significantly lower than that of r-hirudin and LU 58463 ($p < 0.05$) (Figure 2). Heparin is an effective antithrombotic agent. However, when used in the prevention and treatment of arterial and venous thromboembolism, heparin has a number of limitations. Hirudin has a superior antithrombotic effect than that of heparins, but its therapeutic window is quite narrow, the high risk of bleeding being the trade-off for the superior antithrombotic activity.⁹ We confirmed that clot-bound thrombin is enzymatically active, relatively protected from inhibition by heparin but susceptible to AT-independent inhibitors, such as hirudin and LU 58463. The antithrombin effect of LU 58463 in the fluid and in the clot-bound systems is similar to that of hirugen, an exosite I synthetic inhibitor comprising residues 53-64 of the C-terminus of hirudin.¹⁰ The mechanism of the binding of LU 58463 to thrombin is similar to that of the C-terminal domain of hirudin but LU 58463 does not interact with the catalytic site of thrombin. The antithrombotic efficacy of LU 58463 has been demonstrated in animal models, in which this compound induced less bleeding than did hirudin. Whether this lower hemorrhagic effect is due to the sparing of the catalytic site of thrombin by LU 58463 remains to be explored. An additional explanation for the reduced bleeding induced by LU 58463 is that thrombin-induced platelet aggregation is inhibited by LU 58463 at low thrombin concentrations, but is only delayed at higher thrombin concentrations.

We conclude that LU 58453 is more effective than heparin and

as effective as hirudin in inhibiting fibrin-bound thrombin. Due to its mechanism of interaction with thrombin, LU 58453 could be associated with less bleeding complications than hirudin. Therefore, LU 58463 deserves to be tested in the prevention and treatment of thromboembolic diseases as it might be more effective than heparin and safer than hirudin.

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Key words: thrombin, thrombin inhibitors, heparin, hirudin.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. José Antonio Páramo, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Páramo and the Editors. Manuscript received January 9, 2002; accepted August 21, 2002.

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