Comparative Antithrombotic Potencies of Direct Thrombin Inhibitors and Low-Molecular-Weight Heparins in an Ex Vivo Human Experimental Thrombosis Model

Job Harenberg, M.D.,1 Kawei Zokai, M.D.,1 Lukas Piazolo, M.D.,1 Tavidar Fenyvesi, M.D.,1 and Ingrid Jörg, M.D.1

ABSTRACT

Direct thrombin inhibitors (DTIs) such as hirudins and melagatran are currently developed for antithrombotic therapy. They should possess some advantages over the currently used low-molecular-weight heparins (LMWHs). They may also act through an inhibition of thrombin-induced platelet activation. The antithrombotic effects of DTIs and of LMWHs were investigated in an ex vivo thrombosis model with human blood in order to analyze the inhibition of thrombin-antithrombin as well as the platelet factor 4 formation. The data show that DTIs inhibit both fibrin formation and platelet activation, which is of clinical relevance especially for melagatran.

KEYWORDS: Direct thrombin inhibitor, melagatran, low-molecular-weight heparin, platelet factor 4, thrombin formation

Objectives: Upon completion of this article, the reader should be able to (1) list the compounds that were used in this study and (2) summarize the effects that the compounds had on clotting and platelet activation.

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Plasmatic coagulation can be measured by the detection of peptides. These are generated when inactive coagulation factors are transformed into active ones. They are also released from fibrinogen when it is transformed into fibrin through the complex made up of thrombin and antithrombin (TAT) as an intermediary product during the blood coagulation. TAT complexes can be quantified through an enzyme-linked immunosorbent assay (ELISA). Activated platelets release platelet factor 4 (PF4), which can be directly quantified with an ELISA.

We hypothesized that direct thrombin inhibitors (DTIs) exert their antithrombotic potency by inhibition of both thrombin formation and platelet activation as...
soon as blood coagulation is activated. An ex vivo model was used, which reproduced the in vivo situation in man. Endothelial and blood flow influences were not included in this model.

**MATERIAL AND METHODS**

Blood coagulates without any anticoagulation in a plastic tube in 12 to 18 minutes. The plasmatic coagulation can be measured according to the separated products of the activated coagulation factors, which form when the inactive primary stage transforms into the active enzyme system. During the coagulation process, antithrombin forms a complex with thrombin. An ELISA was available (Dade-Behringwerke AG, Marburg, Germany) containing antibodies against antithrombin with which it was possible to quantify the TAT complexes. Reference results were below 4.1 ng/mL. Coagulated blood shows very high TAT results, up to 10,000 ng/mL or more.

PF4 was quantified by means of an ELISA. The reference results were between 1.4 and 6.1 ng/mL. With the coagulation process ex vivo an activation of platelets takes place, as well as the release of PF4 over a period of 18 to 23 minutes.

Free-flowing blood was taken from healthy volunteers (n = 10) from the vena cubitalis with a butterfly needle (1.1 mm) without a tourniquet. After the aspiration, 2 mL of blood was sampled and discarded. After that the blood was sampled in a 10-mL plastic syringe without anticoagulant. The blood was drawn over a period of 30 seconds. Thereafter 0.9 mL of blood was aliquoted into plastic tubes, which already contained the anticoagulant in a ratio of 1:10. The incubation took 30 minutes at 37°C. The coagulation was stopped by adding 0.9 mL of Files Cocktail; the blood was then centrifuged at 3000 g for 10 minutes and the plasma pipetted into a smaller plastic tube. After being shock-frozen with liquid nitrogen and stored at −80°C, the plasma was reprocessed with the TAT ELISA (TAT Enzygnost Micro®, Dade-Behring) and platelet factor 4 ELISA (PF 4 Asserachrom®, Roche Diagnostics, Mannheim, Germany).

As an anticoagulant the following concentration gradients were used: unfractionated heparin (UFH, Braun, Melsungen, Germany), certoparin (Novartis Pharma GmbH, Nuremberg, Germany), dalteparin (Pharmacia & Upjohn, Erlangen, Germany), enoxaparin (Aventis Pharma, Bad Soden, Germany), and nadroparin (Sanofi Synthelabo, Berlin, Germany). The concentrations of the anticoagulants ranged from 0.01 to 3.0 IU/mL. Recombinant (r) hirudin (Aventis Pharma), pegylated (PEG) r-hirudin (Knoll AG, Ludwigshafen, Germany), and melagatran (Astra Zeneca, Mölndal, Sweden) were used in concentrations ranging from 0.1 to 10 μg/mL.

**RESULTS**

Without the addition of an anticoagulant, blood clotted within 30 minutes. The activation of the blood coagulation system resulted in TAT complexes with values of around 10,000 ng/mL. The activation of the platelets was shown in the release of PF4 with values of 9000 ng/mL blood. The results are not listed because the lowest chosen concentration of the anticoagulants did not differ from the concentration zero.

Direct Thrombin Inhibitors

r-Hirudin, PEG-hirudin, and melagatran reduced TAT formation dose dependently in this model. The concentration-dependent inhibition of the TAT complex formation is shown in Figure 1. A 90% inhibition of TAT occurred at 0.15 μg/mL for melagatran, 0.3 μg/mL for PEG-hirudin, and 1.5 μg/mL for r-hirudin.

The inhibitory effect of r-Hirudin, PEG-hirudin, and melagatran on platelet activation is shown in Figure 2. The concentration of PF4 is plotted against the amount of DTI that is added to nonanticoagulated blood ex vivo. A 90% inhibition of PF4 occurred at 0.15 μg/mL for melagatran, 0.3 μg/mL for PEG-hirudin, and 1.5 μg/mL for r-hirudin.

Heparin and LMWHs

UFH and LMWHs had similar effects on TAT formation in the model. A 90% inhibition of TAT generation ranged from 0.15 to 0.35 IU UFH or LMWH, respectively (Fig. 3).

The inhibition of PF4 by UFH and the LMWHs was also similar and differed only to a small extent to the fibrin formation in the concentration ranges. 90% inhibition of PF4 occurred for all compounds between 0.13 and 0.33 IU/mL (Fig. 4).
DISCUSSION

Currently developed antithrombotics apply to direct inhibitors of single proteases of the coagulation system and to inhibitors on platelet stimulation. The DTIs hirudin and argatroban are used to achieve effective anticoagulation in patients with heparin-induced thrombocytopenia without (HIT type I) and with (HITTS or HIT type II) thrombosis. Melagatran is currently under investigation in clinical trials. The results of the present investigation show that melagatran inhibits thrombin generation more effectively than PEG-hirudin or r-hirudin.

Thrombogenesis is quite relevant when it comes to the formation of an arterial thrombosis, such as heart attack, coronary heart disease, unstable angina, or various forms of stroke. The pathogenesis seems to involve largely platelets and their interaction with the vascular wall. Venous thrombogenesis seems to be brought about mostly by the interaction between the plasmatic coagulation system and the endothelium. With all models different techniques cause the thrombosis to be triggered in different ways. These include the infusion of thrombotic factors, toxins, ligation of the vascular system, and implantation of thrombogenic material into the vascular system. More recent techniques were able to show the induction of thrombosis through ultrasound, laser, oxygen radicals, or a photochemical stimulus. The present data demonstrate that DTIs may also exert their antithrombotic action through inhibition of platelet function. Platelets are activated in the present model through thrombin generated during the initiation of blood coagulation and thrombin formation in coagulating blood. Melagatran was more potent than hirudin in inhibiting platelet activation.

The main emphasis in the development of new antithrombotics is on substances that directly affect individual factors of the plasmatic coagulation system and platelets, for example, hirudin, PEG-hirudin, Hirulog, factor Xa-inhibiting peptides, recombinant tissue factor pathway inhibitor, and antibodies against membrane proteins of the platelets. A large number of antithrombotics with possibly better effectiveness or fewer side effects will have to be extensively tried and examined. The results show that in the present model there is a significant dose dependence with clinically relevant dosages of the thrombin inhibitors. In connection with UFH and the LMWHs, the different anti-factor Xa/thrombin ratios did not influence inhibition of thrombin generation and platelet activation. This is assumed to be of relevance for analyzing the ex vivo potency of thrombin and factor Xa inhibitors in the thrombosis model.

Through the use of these markers of the hemostasis system, it is possible to accomplish two things: (1) to establish a differentiated analysis of the involved parts of the plasmatic coagulation system and the platelets in thrombogenesis and (2) to quantify the effects of the antithrombotics. The model also ensures the interaction of substances with effects on blood coagulation.
and platelets. In the field of both substance groups, many new developments have been made.

In summary, we conclude that DTIs such as melagatran and hirudins act through inhibition of thrombin generation as well as platelet activation. A 90% inhibition of both was obtained at physiological concentrations. The different aXa/aIIa ratios of LMWHs were not relevant in this model. This may reflect the clinical efficacy of the chosen doses of the compounds.

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REFERENCES