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In vitro inhibition of thrombin generation, after tissue factor pathway activation, by the oral, direct factor Xa inhibitor rivaroxaban

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Generation of thrombin via the tissue factor (TF) pathway is integral to the blood coagulation process. Therefore, assessment of TF-triggered thrombin generation at different phases of the highly regulated, dynamic process (initiation, propagation, and decay) provides a useful means of studying the inhibitory action of antithrombotic agents [1].

Rivaroxaban (BAY 59-7939; Mr 435.9 Da) is one of a number of novel antithrombotic agents in development that specifically target a single component of the coagulation process. It is an oral, direct factor Xa (FXa) inhibitor (K_i = 0.4 nM), which not only inhibits free FXa but also prothrombinase activity and clot-associated FXa [2,3]. Rivaroxaban demonstrated promising anticoagulant properties in early preclinical studies in human plasma, doubling prothrombin time and activated partial thromboplastin time at concentrations of 230 and 690 nM, respectively [2].

The aim of this study was to investigate the effects of rivaroxaban on each phase of thrombin generation, after activation of the TF pathway, in blood taken from 16 healthy subjects.

Two experimental systems were used to assess thrombin generation in response to the administration of rivaroxaban. Firstly, thrombin generation was assessed indirectly, in whole blood, by measuring prothrombin fragments 1 + 2 (F_{1+2}) – an indicator of prothrombin activation – according to a previously validated method (n = 9) [4]. Second, thrombin generation was assessed directly, in platelet-rich plasma (PRP;

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platelet count adjusted to 300 000 μL^{-1} using autologous platelet-poor plasma), by measuring the cleavage of a fluorogenic substrate (Z-Gly-Gly-Arg-AMC) using a fluorimeter (Fluoroskan Ascent; ThermoLabsystems, Helsinki, Finland) and the Thrombogram-Thrombinoscope® (Synapse b.v., Maastricht, the Netherlands) software. Generated thrombin was quantified using a thrombin calibrator (Thrombogram-Thrombinoscope®) (n = 7). The following parameters were analyzed: (i) the lag time of thrombin generation, (ii) the time taken to reach the maximum concentration of thrombin (ttpeak), (iii) the maximum concentration of thrombin (peak), (iv) the endogenous thrombin potential (ETP), which represents the activity of thrombin multiplied by the time for which it remains active in the plasma, and (v) the rate index of the propagation phase of thrombin generation, calculated by the formula peak/(ttpeak – lag time) and expressed in nM min^{-1}.

In both systems, thrombin generation was triggered in the presence of a minimal concentration of TF (1/3200 final dilution of Hemoliance® Recombiplastin in whole blood; 1/1000 final dilution in PRP). Rivaroxaban was added to whole blood at concentrations ranging from 10 to 5000 nM, and to PRP at concentrations ranging from 5 to 700 nM.

Rivaroxaban prolonged the initiation phase of thrombin generation (represented by the lag time) dose dependently after activation of the TF pathway. At a concentration of approximately 20 nM, rivaroxaban induced a 2-fold increase in the lag time of prothrombin F_{1+2} formation in whole blood; a similar increase in the lag time of thrombin generation was observed with a concentration of 10 nM in PRP. A pronounced inhibitory effect on the propagation phase of thrombin generation was also observed at nanomolar concentrations of rivaroxaban, with IC_{50} values for the rate of prothrombin F_{1+2} formation in whole blood and the rate index of thrombin generation in PRP of 60 and 10 nM, respectively. The inhibitory effects of rivaroxaban on the initiation and propagation phases of thrombin generation were further illustrated by a doubling of the time required to reach the maximum concentration (t_{max}) of prothrombin F_{1+2} in whole blood, at a rivaroxaban concentration of 20 nM. In parallel with this observation, a rivaroxaban concentration of 10 nM was sufficient to double the time required to reach the peak of thrombin generation in PRP (ttpeak).

Furthermore, rivaroxaban reduced the maximum concentration (peak) of thrombin generated, and decreased the ETP in PRP. However, the concentration of rivaroxaban required to induce a 50% reduction in ETP (35 nM) was 3.5-fold higher than that required to double the lag time or halve the rate of thrombin generation (10 nM). Similarly, in whole blood, the concentration of rivaroxaban required to induce a 50% reduction in the C_{max} of prothrombin F_{1+2} was 1000 nM, whereas the concentrations required to double the lag time or halve the rate of prothrombin F_{1+2} formation were approximately 20 and 60 nM, respectively.

Importantly, thrombin generation was almost completely attenuated at high rivaroxaban concentrations: C_{max} of prothrombin F_{1+2} was reduced by > 80% with rivaroxaban 5000 nM in whole blood, and a 90% reduction in ETP was achieved with rivaroxaban 100 nM in PRP.

Figure 1 shows representative curves for prothrombin F_{1+2} formation and thrombin generation in whole blood and PRP, respectively, after activation of the TF pathway.

These data demonstrate that, after activation of the TF pathway in healthy subjects, nanomolar concentrations of the oral, direct FXa inhibitor rivaroxaban significantly prolonged the initiation phase of thrombin generation, and significantly reduced the rate of the propagation phase. As one might expect, these effects led to a reduction in the total amount of thrombin generated, and to a decrease in ETP. Interestingly, the initiation and propagation phases of thrombin generation,

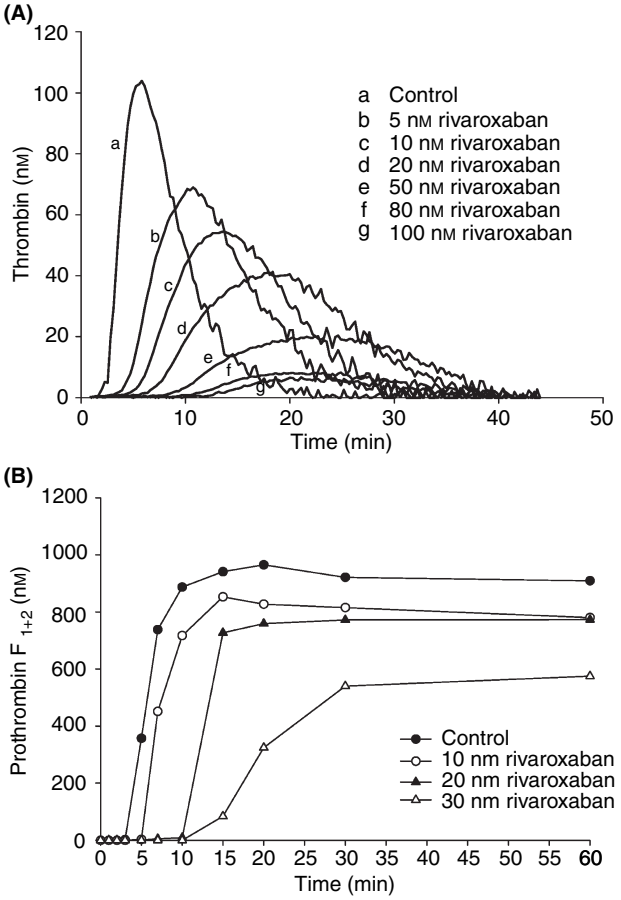

Fig. 1. (A) Representative thrombograms of one of seven experiments showing the influence of rivaroxaban on thrombin generation in platelet-rich plasma (PRP), after activation of the TF pathway. A 20-μL aliquot of diluted recombinant thromboplastin was added to 80 μL of PRP. Thrombin generation was initiated by adding a solution containing $CaCl_2$ (16.7 mM final concentration) and a fluorogenic substrate (417 μM final concentration). (B) Representative curves from one of nine experiments showing the influence of rivaroxaban on the kinetics of prothrombin activation (prothrombin F_{1+2} formation). Prothrombin activation was studied in citrated whole blood (100 μL), after activation of the TF pathway by addition of 25 μL of diluted recombinant human thromboplastin and 25 μL of $CaCl_2$ (0.1 M). At the indicated intervals, 50 μL of serum was mixed with 200 μL of buffer containing ethylenediaminetetraacetic acid, and prothrombin F_{1+2} was measured using an enzyme-linked immunosorbent assay (ELISA) method.

rather than the ETP, were more sensitive to rivaroxaban-induced inhibition of FXa. Given that the ETP describes the cumulative effect of thrombin during the coagulation process as a whole, this suggests that rivaroxaban affects the initiation and propagation phases of thrombin generation to a greater extent than the decay phase. Therefore, parameters representing the initiation or propagation phases may potentially be relevant for determining the antithrombotic activity of rivaroxaban in clinical practice, if this was necessary. Further investigation is, of course, required.

These results are consistent with a previous study of fondaparinux – an indirect antithrombin (AT)-dependent selective FXa inhibitor – which was found to have a more-pronounced effect on the lag time and rate of the propagation phase of thrombin generation than on the ETP [4]. However, unlike fondaparinux, rivaroxaban inhibited thrombin generation almost completely at high concentrations (~5000 nM). The ability of rivaroxaban, but not fondaparinux, to inhibit FXa within the prothrombinase complex provides a plausible explanation for this difference [2,5]. FXa within the prothrombinase complex is protected from inhibition by fondaparinux, perhaps because AT is unable to compete effectively with the substrate prothrombin for the catalytic center of FXa. With its direct, AT-independent mechanism of action, rivaroxaban is able to inhibit free FXa, as well as prothrombinase activity and even clot-associated FXa [2,3]. Therefore, it seems logical that rivaroxaban would exert a greater overall inhibitory effect on thrombin generation than fondaparinux.

Direct thrombin inhibitors have been found to influence thrombin generation in a similar fashion to rivaroxaban, *in vitro* and *ex vivo* [6,7]. However, it can be argued that FXa is superior to thrombin as a target for anticoagulation, because it has fewer functions outside of coagulation, is the primary site of propagation of thrombin generation and activates clotting over a wider concentration range [8]. Comparisons of specific FXa inhibitors with specific thrombin inhibitors in clinical studies would be required to test this hypothesis.

Rivaroxaban demonstrated promising efficacy and safety in double-blind phase II studies for the prevention of venous thromboembolism after major orthopedic surgery [9], and the treatment of acute, symptomatic, proximal deep vein thrombosis [10], and has now entered an extensive phase III program in both indications, as well as in the prevention of stroke in patients with atrial fibrillation.

For the first time, the present study demonstrates that direct FXa inhibition with nanomolar concentrations of rivaroxaban can downregulate and completely suppress the process of thrombin generation in whole blood and PRP. The clinical relevance of this observation requires further investigation.

Disclosure of Conflict of Interests

E. Perzborn is an employee of Bayer HealthCare AG; all authors state that they have no conflict of interest.

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