Effects of the direct thrombin inhibitor dabigatran and its orally active prodrug, dabigatran etexilate, on thrombus formation and bleeding time in rats

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Summary
Dabigatran is a reversible direct, selective thrombin inhibitor, undergoing clinical development as its orally active prodrug, dabigatran etexilate. The objective of this trial was to assess the antithrombotic and anticoagulant effects of dabigatran and dabigatran etexilate in a rat model of venous thrombosis. In order to do this a modified Wessler model was used to assess the antithrombotic and anticoagulant effects of intravenous (i.v.) dabigatran and oral dabigatran etexilate administration. In addition, a rat tail bleeding time model was used to investigate the antihemostatic effect of dabigatran. The study demonstrated that bolus administration of dabigatran (0.01–0.1 mg/kg) reduced thrombus formation dose-dependently, with an ED\textsubscript{50} (50% of the effective dose) of 0.033 mg/kg and complete inhibition at 0.1 mg/kg. By comparison, ED\textsubscript{50} values for heparin (0.03–0.3 mg/kg), hirudin (0.01–0.5 mg/kg) and melagatran (0.1–0.5 mg/kg) were 0.07, 0.15 and 0.12 mg/kg, respectively. Oral administration of dabigatran etexilate (5–30 mg/kg) inhibited thrombus formation in a dose- and time-dependent manner, with maximum inhibition within 30 min of pretreatment, suggesting a rapid onset of action. Following i.v. administration of dabigatran (0.1–1.0 mg/kg), a statistically significant prolongation of bleeding time was observed at doses at least 15- and 5-fold greater than ED\textsubscript{50} and ED\textsubscript{100} (100% of the effective dose) doses, respectively; there was no significant increase in bleeding tendency at the maximum therapeutically effective dose (0.1 mg/kg). It can be concluded that dabigatran and its oral prodrug, dabigatran etexilate, show promise in the management of thromboembolic disease.

Keywords
Activated partial thromboplastin time, anticoagulant, dabigatran etexilate, direct thrombin inhibitor, thrombosis

Introduction
Unfractionated heparin (UFH), low-molecular-weight heparins (LMWHs) and, more recently, fondaparinux, are standard therapies for the immediate prophylaxis and treatment of deep vein thrombosis (DVT), whilst the vitamin K antagonists (e.g. warfarin) are the only oral anticoagulants available so far for the long-term treatment of DVT and the prevention of stroke in patients with atrial fibrillation (1–3).

Although the use of these agents has led to a considerable reduction in the incidence of thromboembolic complications, they are associated with a number of limitations. In particular, heparin and LMWH are suitable only for parenteral administration, whilst warfarin has a narrow therapeutic window, requires regular coagulation monitoring and is associated with drug and food interactions (2, 3).

These limitations have encouraged the development of new anticoagulants, designed to target specific steps in the coagulation system (4).

Thrombin is a trypsin-like serine protease that is a key factor in coagulation, and plays a central role in thrombogenesis (5). Whereas indirect thrombin inhibitors, such as UFH and LMWH, act by enhancing thrombin inhibition by endogenous antithrombin (2), direct thrombin inhibitors (DTIs) bind directly to thrombin (6). Since DTIs can inactivate both fibrin-bound and fluid-phase thrombin, they prevent thrombus formation more effec-
tively than indirect thrombin inhibitors (7, 8); they also exhibit a more predictable anticoagulant effect (9).

Ximelagatran, the prodrug of melagatran, was the first orally administered DTI which showed convincing antithrombotic efficacy in Phase III trials (10). However, although it was initially registered in Europe for the short-term indications of venous thromboembolism (VTE) prevention, its development has ultimately been stopped due to long-term safety concerns (11).

Dabigatran (BIBR 953 ZW) is a synthetic DTI with a high affinity and specificity for thrombin (12, 13). Its orally active prodrug, dabigatran etexilate (BIBR 1048 MS), is currently undergoing phase III clinical development for the prophylaxis and treatment of VTE and the prevention of stroke due to atrial fibrillation. The aim of the present study was to explore the full dose range of the antithrombotic effects of dabigatran in a rat model of venous thrombosis after single intravenous (i.v.) administration, and to compare these effects with those of already established anticoagulants, namely UFH, hirudin and melagatran. We also determined the anti-haemostatic effects of dabigatran after single i.v. administration in a rat template bleeding time model in order to explore a potential window between the maximum antithrombotic effects and bleeding tendency in this animal model. Finally, dabigatran etexilate was administered to conscious rats by oral gavage at doses known to produce significant anticoagulant effects in order to demonstrate the dose- and time-dependent antithrombotic efficacy of the prodrug.

Methods

Preparation of test solutions

Dabigatran

For i.v. administration, dabigatran (Boehringer Ingelheim Pharma KG, Biberach, Germany) was dissolved (10 mg/ml) in 1% dimethylsulfoxide (DMSO) and diluted with 99% physiological saline. In the rat bleeding time study, the compound was dissolved (10 mg/ml) in 1% DMSO, 30% glycerol-formal-cremophore (GFC), 0.5% of a 0.1 N hydrogen chloride (HCl) solution and 68.5% H2O. This stock solution was diluted to the required concentration with physiological saline and administered intravenously at a final volume of 1.0 ml/kg body weight.

Dabigatran etexilate

Doses of dabigatran etexilate (Boehringer Ingelheim Pharma KG, Biberach, Germany) for oral administration were prepared freshly as aqueous solutions containing 1% (vol/vol) DMSO and 30% (vol/vol) GFC. Control animals received the treatment vehicle without the active agent.

Hirudin

Hirudin (Refludan™; lyophilized recombinant lepirudin for injection; Hoechst Marion Roussel, Germany) was reconstituted in Tris buffer (10 mg/ml) and diluted in 0.9% physiological saline.

Melagatran

Melagatran (synthesized in the Dept. Medicinal Chemistry at Boehringer Ingelheim Pharma GmbH & Co KG, Biberach, Germany) was dissolved and diluted in 0.9% physiological saline.

UFH

Heparin sodium (Ratiopharm, Ulm, Germany) was diluted with 0.9% physiological saline.

Induction of venous thrombosis in rats

All experimental procedures were approved and conducted in accordance with the German Animal Protection Act (Deutsches Tierschutzgesetz). A rat model of VTE using stasis and tissue thromboplastin to achieve hypercoagulability (Wessler model), as described by Herbert et al. (14), was employed with slight modifications. Male rats (Chbb Thom, 280–330 g) were anesthetized with sodium pentobarbitone (Rhone Merieux, Laupheim, Germany; 60 mg/kg intraperitoneally; 0.1 ml/100 g body weight), and the right jugular vein was cannulated for i.v. injection of thromboplastin and study drug. The right carotid artery was cannulated to collect blood samples. The abdominal caval vein was exposed for the placing of vessel clamps, and small side branches were ligated. Thrombus formation was induced 15 minutes (min) after induction of anesthesia by the injection of human placental thromboplastin (Thromborel S [Dade Behring, Marburg, Germany]) diluted 1:10 with saline; 0.1 ml/100 g body weight), followed 30 seconds (s) later by blood stasis in a 1.5 cm segment of the caval vein. Stasis was maintained for 10 min, and the thrombus was then removed, dried and weighed.

Pretreatment of animals

Dabigatran/reference thrombin inhibitors

Dabigatran and the reference thrombin inhibitors, or the respective treatment vehicles, were administered as an i.v. bolus 5 min before the injection of thromboplastin. The doses administered were:

- Dabigatran: 0.01, 0.03, 0.05 and 0.1 mg/kg body weight
- UFH: 0.033, 0.067, 0.2 and 0.33 mg/kg body weight
- Hirudin: 0.01, 0.1, 0.3 and 0.5 mg/kg body weight
- Melagatran: 0.03, 0.1 and 0.3 mg/kg body weight

Blood (0.45 ml) was withdrawn using 3.8% trisodium citrate (1 volume of citrate for 9 volumes of blood) as anticoagulant. Samples were collected prior to and 5 min after i.v. administration of the study drugs or treatment vehicles and stored on ice prior to determination of activated partial thromboplastin time (aPTT).

Dabigatran etexilate

Male rats (Chbb Thom, 280–330 g) were fasted overnight before receiving a single oral dose of dabigatran etexilate or treatment vehicle via a gastric tube. Dabigatran etexilate was administered at doses of 5, 10, 20 or 30 mg/kg body weight. Each dose group was subdivided according to pretreatment period in order to investigate time-dependent antithrombotic effects. The pretreatment period was defined as the period between the administration of the compound and the induction of anesthesia. Pretreatment periods of 0.5, 1, 2, 3, and 5 hours (h) were employed in all active treatment groups, and an additional 7 h pretreatment period was employed in the two highest dose groups. Control animals were pretreated for 1 h, since preliminary experiments demonstrated that pretreatment periods below and beyond 1 h did not influence either thrombus weight or aPTT (data not shown). A 0.45 ml blood sample was collected from each animal...
immediately prior to the injection of thromboplastin and stored in citrate buffer, for the determination of aPTT.

**Determination of rat tail bleeding time**

A rat tail incision bleeding model, as described by Gustafsson et al. (15), was employed with slight modifications. Male rats (Cabb Thom, 200–220 g) were anesthetized with sodium pentobarbital (Rhone Merieux, Laupheim, Germany; 60 mg/kg intraperitoneally; 0.1 ml/100 g body weight), and the right jugular vein was cannulated for the i.v. administration of dabigatran or treatment vehicle. Dabigatran was administered at doses of 0.1, 0.3, 0.5 or 1.0 mg/kg body weight. These doses were selected because they represent the fully effective (0.1 mg/kg) and supra-maximal doses of dabigatran in the rat venous thrombosis model. Bleeding time was measured using a spring-loaded blade device (Surgicutt International, Technidyne Corp, Edison, NJ, USA) that was applied longitudinally on the surface of the tail, 9 cm from the tip, avoiding large veins. Blood emerging from the incision was gently wiped away every 15 s. Bleeding time (defined as time elapsed until bleeding stopped) was measured prior to, and 15, 30, 45, 60, 90 and 120 min after the i.v. administration of dabigatran or vehicle. With the exception of the highest dabigatran dose group, each animal served as its own control. In the highest dose group, the bleeding time at early time points exceeded the pre-defined time intervals, thus three separate groups were run in parallel, with the bleeding time measurements conducted 15, 30 and 45 min after dabigatran administration.

**Analytical methods**

Activated partial thromboplastin time (PTT)

Activated PTT was measured in a coagulometer (Biomatic B10, Sarstedt, Germany) using PTT reagent (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. From each native citrated blood sample, 0.1 ml was pipetted into a test tube pre-warmed to 37°C. PTT reagent (0.1 ml) was added, and the solution was mixed and incubated for 3 min. Calcium chloride solution (0.1 ml), pre-warmed to 37°C, was added in order to activate the coagulation cascade, and aPTT was determined as the time elapsed between the addition of calcium chloride solution and the onset of clotting.

**Statistics**

All data are expressed as mean ± standard error of the mean (SEM). For experiments investigating the i.v. administration of dabigatran and reference thrombin inhibitors, calculation of the ED$_{50}$ for inhibition of clot dry weight was determined from the linear section of the dose-response curve of each compound. Linear regression analysis was used to determine 95% confidence intervals (CI) for each compound’s ED$_{50}$. For experiments investigating the oral administration of dabigatran etexilate, Dunnett’s test was used to compare thrombus dry weight and aPTT between animals administered active treatment and those administered treatment vehicle. For experiments investigating bleeding times, baseline parameters in different treatment groups were compared using one-way analysis of variance and Tukey’s test; Dunnett’s test was used to compare treated groups with their respective time-matched controls. A p-value of <0.05 was considered statistically significant for all tests.

![Figure 1: Dose-response curve for the antithrombotic efficacy of dabigatran (0.01–0.1 mg/kg), as measured by the reduction in clot weight in the rat venous thrombosis model. Average dry clot weight in the vehicle-treated group was 10.3 ± 1.0 mg. Values are presented as mean ± SEM of 4–6 animals/group. * = p<0.05; **=p<0.01 versus control clot weight](image)

**Results**

Antithrombotic and anticoagulant effects of dabigatran in comparison with reference thrombin inhibitors

The dose-dependency of the antithrombotic effect of dabigatran following i.v. administration is shown in Figure 1. In this Wessler model, dabigatran inhibited clot formation with an ED$_{50}$ of 0.033 mg/kg. Complete inhibition was achieved with the highest dose. Comparative efficacy data from the respective reference compounds are presented in Table 1. Thus, the potency order in terms of the ED$_{50}$ values of the compounds tested was dabigatran > UFH > melagatran > hirudin. In addition, administration of dabigatran and the three reference thrombin inhibitors prolonged the aPTT in a dose-dependent manner. An inverse correlation between the decrease in thrombus weight, and an increase in aPTT was observed following administration of dabigatran and the three comparator drugs (Fig. 2).

**Antithrombotic and anticoagulant effects of dabigatran etexilate**

The thrombus dry weight in the vehicle-treated animals amounted to 9.04 ± 0.75 mg. Oral pretreatment of rats with dabigatran etexilate at varying doses (5–30 mg/kg) for periods ranging from 30 min to 7 h prior to induction of anesthesia revealed a dose- and time-dependent inhibition of clot formation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose range (mg/kg)</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dabigatran</td>
<td>0.01–0.1</td>
<td>0.033</td>
<td>0.012–0.056</td>
</tr>
<tr>
<td>UFH</td>
<td>0.03–0.3</td>
<td>0.073</td>
<td>0.062–0.087</td>
</tr>
<tr>
<td>Hirudin</td>
<td>0.01–0.5</td>
<td>0.152</td>
<td>0.059–0.246</td>
</tr>
<tr>
<td>Melagatran</td>
<td>0.03–0.3</td>
<td>0.122</td>
<td>0.076–0.168</td>
</tr>
</tbody>
</table>

ED$_{50}$ values and 95% CI were calculated from the linear part of the dose-response curve.
Maximum inhibition of clot formation was achieved within 30 min of pretreatment, suggesting a rapid onset of action following oral administration. Significant inhibition of clot weight was still observed after 2 h in the lowest dose group and after 3 h in all higher dose groups.

Dose- and time-dependent prolongation of aPTT was also observed after oral administration of dabigatran etexilate, with the three higher doses showing significant prolongation after 0.5–3 h of pre-treatment (Table 3). The aPTT returned to baseline levels after 5 and 7 h, respectively.

**Rat tail bleeding time**

Rat tail bleeding time was assessed after i.v. administration of dabigatran at doses of 0.1–1.0 mg/kg (Table 4). The two lower doses (0.1 and 0.3 mg/kg) did not significantly prolong bleeding time although a trend towards an increase was observed with the 0.3 mg/kg dose. In contrast, both higher doses (0.5 and 1.0 mg/kg) induced a statistically significant increase in bleeding time. These higher doses are 5– and 10-fold higher, respectively, than the maximum effective antithrombotic dose (0.1 mg/kg).

<table>
<thead>
<tr>
<th>Pretreatment period (h)</th>
<th>Thrombus dry weight (mg) [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>0.5</td>
<td>1.7 ± 0.7 [6]**</td>
</tr>
<tr>
<td>1</td>
<td>3.4 ± 1.0 [6]**</td>
</tr>
<tr>
<td>2</td>
<td>5.3 ± 1.0 [6]**</td>
</tr>
<tr>
<td>3</td>
<td>6.8 ± 0.9 [8]</td>
</tr>
<tr>
<td>5</td>
<td>8.2 ± 0.9 [6]</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM, with number of animals in parentheses. *p<0.05 and **p<0.01, compared with vehicle-treated group (9.04 ± 0.75 mg; n=10). ND = not determined.
Table 3: Anticoagulant effect, as measured by aPTT, after single oral administration of dabigatran etexilate at four doses (5, 10, 20 and 30 mg/kg) for different pretreatment periods (0.5–7 h).

<table>
<thead>
<tr>
<th>Pretreatment period (h)</th>
<th>5 mg/kg</th>
<th>10 mg/kg</th>
<th>20 mg/kg</th>
<th>30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>47 ± 7 [6]**</td>
<td>67 ± 5 [6]**</td>
<td>146 ± 10 [6]**</td>
<td>271 ± 24 [6]**</td>
</tr>
<tr>
<td>1</td>
<td>23 ± 1 [6]*</td>
<td>52 ± 4 [6]**</td>
<td>98 ± 14 [6]**</td>
<td>155 ± 20 [6]**</td>
</tr>
<tr>
<td>2</td>
<td>18 ± 1 [6]</td>
<td>23 ± 1 [6]*</td>
<td>47 ± 8 [8]**</td>
<td>100 ± 14 [6]**</td>
</tr>
<tr>
<td>3</td>
<td>15 ± 0.7 [8]</td>
<td>18 ± 0.3 [6]</td>
<td>25 ± 2 [8]</td>
<td>32 ± 2 [6]**</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>13 ± 0.2 [6]</td>
<td>14 ± 1 [6]</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S EM, with number of animals in parentheses. *p<0.05 and **p<0.01, compared with vehicle-treated group (13.3 ± 1.0 s; n=10). ND=not determined.

Table 4: Effect of dabigatran (0.1–1.0 mg/kg) on rat tail bleeding time at different time points after i.v. administration.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tail bleeding time (s) [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-dose</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S EM, with number of animals in parentheses. *p<0.05, compared with time-matched vehicle-treated group.

Discussion

In the present study, we have chosen a rodent model of venous thrombosis that has been frequently used for the characterization of the antithrombotic efficacy of different novel anticoagulants (15–18). In this model, administration of i.v. dabigatran was shown to reduce thrombus formation in a dose-dependent manner, with complete inhibition achieved at a dose of 0.1 mg/kg. Since antithrombotic effect was measured after a relatively short period of time, with induction of thrombus formation 5 min after the administration of the test compound, pharmacokinetic variability between the antithrombotic agents tested was minimized, allowing direct comparison. Antithrombotic efficacy of dabigatran was well in the range of all the comparator agents investigated, as shown by ED₅₀ values in the following order: dabigatran > UFH > melagatran > hirudin. The antithrombotic effects of the comparator agents used in this study have previously been described in various models of thrombosis (19, 20). Whereas dabigatran and melagatran are potent, competitive and reversible active site inhibitors of thrombin, hirudin has a very high affinity for thrombin, with a relatively slow onset of action (21). This may partly explain the relatively weak potency it demonstrated in the present study, compared with the synthetic inhibitors.

For dabigatran and the comparator agents, the observed dose-dependent inhibition of thrombus formation was directly correlated with a prolongation of aPTT. The second sampling point for aPTT measurement was immediately prior to the administration of thromboplastin, reflecting plasma drug levels at the time of liberation, thereby demonstrating the maximal anticoagulant activity obtained in the segment of caval vein at the time of stasis induction. For dabigatran, as for each agent, aPTT increased dose-dependently and an inverse correlation between aPTT prolongation and reduction in clot weight could be shown, an effect similar to that observed by others (18, 20). This implies a direct correlation between the anticoagulant and antithrombotic effects for all agents used in this model.

The aPTT is widely used to monitor heparin therapy and has served as surrogate efficacy marker in the clinical development of several new anticoagulants (22, 23). However, the aPTT may vary considerably according to the reagent and coagulometer used (22, 24). Moreover, the concentration of a thrombin inhibitor needed to double the aPTT in whole blood or plasma can differ considerably from one species to the other (25). Therefore, the range obtained for the aPTT as a surrogate marker for antithrombotic efficacy in our rat thrombosis model may not precisely reflect the therapeutically effective range in the clinical setting.

Similar antithrombotic and anticoagulant properties were observed with the oral administration of the prodrug, dabigatran etexilate, which was shown to inhibit thrombus formation and increase the aPTT in a dose- and time-dependent manner. Notably, both lower doses of 5 and 10 mg/kg, which already showed convincing antithrombotic efficacy, approximate the clinically effective doses in the prophylaxis of thrombosis in orthopedic surgery investigated in the clinical development program (26, 27). Thrombus formation was significantly reduced with all doses of dabigatran etexilate after 30 min of pretreatment, demonstrating...
a rapid absorption phase and a fast onset of action. The almost immediate onset and reversible mode of anticoagulant and antithrombotic efficacy of dabigatran, thus offers a significant advantage over warfarin, which usually has to be dosed for at least three to five days in order to reach steady-state antithrombotic efficacy in experimental or clinical settings (3, 20).

We investigated bleeding times in a well characterized experimental model (15, 16) that measures bleeding from small vessels, in order to distinguish antithrombotic from antihemostatic effects of dabigatran. A statistically significant prolongation of bleeding time was observed with dabigatran only at doses that were at least 15- and 5-fold higher than the ED50 and ED100 doses, respectively (i.e. at doses ≥0.5 mg/kg). Importantly, the 0.1 mg/kg dose, which was shown to be a fully effective antithrombotic dose in this species, did not significantly prolong bleeding time. A similar dissociation between the antithrombotic efficacy and prolongation of bleeding time has also been reported by others, using either orally active thrombin inhibitors, or FXa inhibitors (28–30). However, despite its widespread use, the extrapolation of this bleeding time model to the clinical setting should be performed with caution (31).

In conclusion, our data show that dabigatran – administered as either its active form or its prodrug – achieves potent, dose-dependent anticoagulant and antithrombotic effects that are comparable to those of known, efficacious anticoagulants. The antithrombotic efficacy of dabigatran demonstrated good correlation with its anticoagulant effects. These findings suggest that dabigatran shows promise in the management of thromboembolic disease.

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References