The direct thrombin inhibitor argatroban effectively prevents cardiac catheter thrombosis in vitro

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Summary
The direct thrombin inhibitor argatroban offers some significant advantages over unfractionated heparin (UFH) and is recommended as an alternative anticoagulant during percutaneous coronary interventions (PCI). The impact of argatroban on cardiac catheter thrombosis — a severe potential complication of PCI — has not been systematically studied yet. The aim of the present study was to test in vitro the hypothesis that argatroban is equivalent to the more established anticoagulants UFH and enoxaparin in preventing catheter thrombus formation. Blood pretreated with the anticoagulants of interest was continuously circulated through a guiding catheter by using a roller pump for a maximum experimental period of 60 minutes. In an alternate model, coagulation was mechanically induced by a magnetic stirrer. Coagulation parameters, overall thrombus weight and electron microscopic features (deposits of platelets and fibrin on the catheter surface) were quantified as endpoints. Argatroban (administered as bolus or continuous infusion), UFH (bolus), and enoxaparin (bolus) significantly reduced catheter thrombus formation compared to untreated controls. Here, neither overall thrombus weight nor platelet/fibrin deposition was different among the specific anticoagulants. Declining ACT (activated clotting time) levels — which were found in the argatroban bolus group — could be prevented by continuous infusion. In magnetic stirrer-induced coagulation, thrombus weight was lower following bolus treatment with UFH and enoxaparin compared to argatroban. These data suggest that the potential for argatroban in preventing catheter thrombosis is comparable to that of UFH and enoxaparin. However, the anticoagulatory efficacy varied, depending on the model of coagulation activation, which demonstrates the necessity for specific testing.

Keywords
Anticoagulation, direct thrombin inhibitors, PCI

Introduction
Systemic anticoagulants represent a cornerstone in the contemporary medical management of acute coronary syndromes (ACS) (1–3). However, unfractionated heparin (UFH) — which since its discovery in 1916 remains the most frequently employed anticoagulant in ACS — exhibits important drawbacks, such as platelet activation, rebound phenomena, or immunogenic complications [e.g. heparin-induced thrombocytopenia (HIT)], which have stimulated the development of more effective and safer antithrombin agents (4, 5).

Direct thrombin inhibitors (DTI) constitute a recently developed class of antithrombin agents which target the thrombin molecule as a central factor of the coagulation cascade (6). In contrast to UFH, DTI inhibit thrombin directly — i.e. without the presence of antithrombin being indispensable — and regardless of whether thrombin is soluble or bound to fibrin. DTI do not stimulate platelet activation and aggregation, which may be regarded as a crucial advantage over heparins, especially in the context of ACS (4). Furthermore, due to a low immunogenic potential and their structural difference from heparins, DTI represent a valuable alternative to heparins in the context of HIT (7). Consequently, contemporary guidelines recommend the DTI bivalirudin or argatroban as alternatives to heparin during percutaneous coronary interventions (PCI), especially with respect to HIT (8, 9).

Argatroban, a synthetic DTI derived from L-arginine, reversibly binds the active site of thrombin and prevents fibrin formation and platelet aggregation without having an inhibitory effect on other serine proteases at therapeutic concentrations (10). Indeed, argatroban exhibits predictable pharmacodynamic responses, as measured by either the activated partial thromboplastin time (aPTT) or activated clotting time (ACT), and is therefore increasingly being employed in PCI for ACS (11–13). In contrast to ximelagatran, an orally available DTI which was withdrawn from the

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market due to an increased risk of liver toxicity after prolonged administration, argatroban seems to be safe and moreover – after proper dose adjustment – may provide safe and effective antithrombotic therapy in patients with hepatic impairment (14, 15).

With respect to PCI, clinical investigation of modern anticoagulants in ACS has revealed another important aspect of antithrombin therapy, namely, the prevention of thrombus formation on the cardiac catheter equipment (i.e. catheter thrombosis). For example, the OASIS-5 and OASIS-6 trials revealed an increased incidence of catheter thrombosis in patients with ACS who received the selective factor Xa inhibitor fondaparinux for systemic anticoagulation (16, 17).

As catheter thrombus formation can result in disastrous consequences during PCI, our group has focused on this particular issue of anticoagulation using an in vitro model. Given the therapeutic significance of PCI, all anticoagulants employed or tested in the setting of ACS should also effectively prevent catheter thrombosis formation.

The aim of the present study was to test in vitro the hypothesis that argatroban is equivalent to the more established anticoagulants UFH and enoxaparin in preventing catheter thrombus formation.

**Materials and methods**

**Blood sample preparation and experimental processing (cardiac catheter)**

Blood (50 ml) from 10 healthy male donors was used for each anticoagulatory condition investigated, whereby donors served as their own control. All blood donors took 500 mg aspirin orally 2 hours (h) before blood was drawn.

The blood was venedected directly into a 50 ml tube which was prepared with the specific anticoagulants of interest, namely: 1) no anticoagulant (control); 2) argatroban – bolus 3 μg/ml + eptifibatide 1.7 μg/ml; 3) argatroban – infusion (3 μg/ml + 2.5 μg/ml/h); 4) argatroban – infusion + eptifibatide; 5) enoxaparin 0.6 U/ml; 6) enoxaparin + eptifibatide; 7) UFH 0.8 U/ml; and 8) UFH plus eptifibatide. The concentrations of enoxaparin and UFH were adopted from previous studies (18, 19) as they were found effective to induce levels of anti-Xa activity (enoxaparin) and ACT (UFH) as recommended for PCI (9, 20). The concentration of argatroban was determined empirically to reach ACT levels appropriate for PCI (7, 9, 21). Subsequently, experiments were performed as described previously (18). Briefly, blood from the tube (preserved in a 37°C water bath) was circulated via a 6F multipurpose guiding catheter (Cordis®) by a roller pump (Masterflex®) at a flow rate of 3 ml/minute (min) for a maximum experimental duration of 60 min. If the catheter was occluded before 60 min of circulation had elapsed, this time was recorded. Additional experiments without catheter perfusion were performed.

For a more detailed analysis of thrombus formation, the degree of platelet and fibrin deposits on the catheter surface was determined by electron microscopy. For that purpose catheters were prepared as described previously (18). Briefly, catheters were cleansed of blood using Soerensen solution that was introduced into the circulation for approximately 60 seconds (sec). Catheter-adherent thrombi were then fixed by glutaraldehyde. Subsequently, catheter tips (1 cm) were cut longitudinally and then dehydrated in a graded series of acetone solution, treated with hexamethyldisilazane to prevent oxidation, and dried overnight under an exhaust system.

For the scanning electron microscopic analysis, a LEO 1530 scanning electron microscope (Gatech®, Georgia Institutes of Technology, Atlanta, GA, USA) was used. A 100x150 μm region central to the catheter tips was photographed and, subsequently, the number of platelets in this area was counted. Fibrin deposition was quantified according to a scale from 0 – 4 (0= no fibrin deposition, 1= trace fibrin deposition, 2= low fibrin deposition, 3= moderate fibrin deposition, and 4= heavy fibrin deposition). All microscopic analyses were undertaken by two independent investigators blinded to the anticoagulant used.
Determination of coagulation parameters (ACT, anti-Xa activity, T-AT)

To determine and compare the respective anticoagulation levels ACT was quantified by using a Medtronic ACT PLUS® device and anti-Xa activity was detected by using the routine laboratory methods employed at Martin Luther-University Halle-Wittenberg, Germany. T-AT was quantified as a measure of thrombin generation using the ENZYGNOST TAT micro ELISA (Dade Behring, Marburg, Germany).

Statistics

One-way ANOVA with post-hoc analysis was performed for comparisons between experimental groups. P-values <0.05 were considered to be statistically significant.

Results

Catheter thrombus weight and catheter circulation time

Thrombus weight was taken as the overall quantitative marker of thrombus formation under the different anticoagulants investigated. Additionally, catheter circulation time was recorded as a gross indicator of thrombus formation that leads to complete catheter occlusion.

Blood that had not been treated with an anticoagulant (serving as control) completely occluded the catheter after an average of 5.8 ± 1.1 min, whereas for all anticoagulants tested the catheter remained patent for the entire or nearly entire experimental time of 60 min (argatroban – bolus + eptifibatide: 54.7 ± 12.6 min; argatroban – infusion: 59.2 ± 1.9 min; argatroban – infusion + eptifibatide: 59.8 ± 0.6 min; UFH: 60 ± 0 min; UFH + eptifibatide: 60 ± 0 min; enoxaparin: 59.4 ± 2 min; enoxaparin + eptifibatide: 60 ± 0 min).

Thrombus weight was significantly lower in all anticoagulant groups than in untreated controls (Fig. 1). However, no statistically significant difference was observed among the specific anticoagulants tested (Fig. 1). Additional treatment with eptifibatide did not affect thrombus weight regardless of whether it was administered in combination with argatroban, UFH, or enoxaparin (Fig. 1).

Parameters of anticoagulation

Anti-Xa activity – which was assessed to monitor low-molecular-weight heparin-induced anticoagulation – was not significantly different between enoxaparin (0.77 ± 0.18 IU/ml) and enoxaparin + eptifibatide (0.81 ± 0.06 IU/ml) regimen.

ACT was used as a parameter of anticoagulation achieved by argatroban and UFH treatment. Here, we found no difference between the individual treatment strategies using argatroban or UFH.
(Fig. 1). However, ACT was lower in the UFH groups than for the argatroban regimen (Fig. 1).

Comparison of the ACT time course between the argatroban bolus and argatroban infusion regimen showed that ACT was significantly lower in the bolus group than under infusion conditions, where ACT levels remained constant (Fig. 2). To investigate presumed thrombin-related argatroban consumption as an explanation for these declining ACT levels, thrombin-antithrombin complexes (T-AT) were quantified as a measure of thrombin generation. Here we found plasmatic T-AT levels significantly increasing during the experimental course (Fig. 3).

**Electron microscopy studies**

Electron microscopy was performed to evaluate catheter-associated thrombus formation in more detail.

Fibrin deposition on the catheter tips did not significantly differ for any of the specific anticoagulant treatments tested (argatroban – bolus + eptifibatide: 1.1 ± 0.6; argatroban – infusion: 2.2 ± 1.5; argatroban – infusion + eptifibatide: 1.1 ± 0.6; UFH: 1.0 ± 0.6; UFH + eptifibatide: 1.0 ± 0.2; enoxaparin: 2.2 ± 1.6; enoxaparin + eptifibatide: 1.8 ± 1.0). At the same time, the number of platelets adherent to the catheter was similar (i.e. not significantly different; argatroban – bolus + eptifibatide: 389 ± 110; argatroban – infusion: 226 ± 203; argatroban – infusion + eptifibatide: 211 ± 162; UFH: 339 ± 225; UFH + eptifibatide: 201 ± 116; enoxaparin: 121 ± 86; enoxaparin + eptifibatide: 341 ± 267.

**Magnetic stirrer**

The magnetic stirrer was employed as an alternative model of coagulation activation under mechanically challenging conditions.

Investigating the different anticoagulants following bolus administration we qualitatively (photographic thrombus aspect; Fig. 4B) and quantitatively (stirrer weight; Fig. 4A) found that thrombus generation was lower under UFH and enoxaparin treatment compared to argatroban.

**Discussion**

DTIs are emerging as a promising class of anticoagulants in the management of acute coronary syndromes. Data from the REPLACE-2 trial and the ACUITY trial have established that bivalirudin, a member of the DTI family, is a safe and effective agent that – compared to heparins – reduces major bleedings without increasing thrombotic events in both lower- and higher-risk patients undergoing PCI (22, 23).

Argatroban, a synthetic DTI with a distinct pharmacologic profile different from that of bivalirudin (24, 25) – lower affinity to thrombin (Ki=40 nM vs. Ki=2 nM), different mode of elimination (hepatobiliary excretion vs. non-organ-dependent proteolysis) – is another agent increasingly being employed during PCI, especially in patients with a history of or with acute HIT (11, 26).

Cardiac catheter thrombosis is a rare but potentially disastrous complication in the context of PCI (27). As this aspect of anticoagulation is highly controversial – reports from recent clinical studies range from sufficient antiplatelet-only regimen without systemic anticoagulation for simple elective PCI to a superiority of UFH over enoxaparin (28, 29) – our group sought to focus in particular on catheter thrombosis using an in vitro approach. Having developed a sensitive model of cardiac catheter thrombosis we were able to reproduce, at the in vitro level, the higher incidence of catheter thrombosis following anticoagulation with fondaparinux initially reported from clinical trials (18).
As clinical data concerning argatroban usage in PCI as compared to bivalirudin are limited, the present study was designed to test the hypothesis that argatroban is equivalent to UFH and enoxaparin in preventing catheter thrombus formation.

Here, we found that all anticoagulants tested (argatroban, UFH, and enoxaparin) significantly reduced catheter thrombus formation in comparison to controls without anticoagulant treatment – once again demonstrating the necessity of employing anticoagulants to counteract catheter surface thrombogenicity. Although we observed some slight differences concerning total circulation time, our catheter model demonstrated a comparable efficacy of all anticoagulatory conditions tested with regard to overall thrombus weight and microscopic features of thrombus formation, such as catheter-adherent platelets or fibrin deposits.

Of note is that the initial ACTs – which all reached levels appropriate for PCI – were higher in the groups employing argatroban than under UFH-treated conditions (Fig. 1). However, it is known that ACTs obtained by the Medtronic ACT PLUS® device (HemoTec system) – which was used in this study – are relatively higher under argatroban medication and lower under heparin treatment as compared to other monitoring devices (30). In this respect target ACT level recommendations for PCI are different for UFH (250–300 sec by HemoTec) and argatroban (300–450 sec, regardless of the device) (7, 9, 21). Furthermore, under argatroban given as a bolus medication the declining ACT levels – as quantified after 30 and 60 min (Fig. 2) – still prevented the formation of thrombi in the catheter (Fig. 1).

In contrast to our previous data investigating the effects of bivalirudin on cardiac catheter thrombosis, argatroban inhibited thrombus formation regardless of whether it was administered as a bolus or continuously (19). This finding might be explained by the different modes of DTI elimination: bivalirudin is cleaved by organ-independent proteolysis, whereas argatroban is excreted via the hepatobiliary system, which obviously is not present in an in vitro setting (24). Nevertheless, our temporal ACT analyses (Fig. 2) revealed a decline in an argatroban-related effect, suggesting in vitro drug elimination. This phenomenon might be explained on a

What is known about this topic?
- In interventional cardiology sufficient anticoagulation is critical for the prevention of cardiac catheter thrombosis as a potentially severe complication during percutaneous coronary interventions (PCI).
- The direct thrombin inhibitor argatroban is recommended as alternative to heparin during PCI, especially in patients with heparin-induced thrombocytopenia (HIT).
- In the light of a variable potential among different types of anticoagulants to prevent cardiac catheter thrombosis the anticoagulatory profile of argatroban has not been systematically evaluated yet.

What does this paper add?
- In our in vitro study argatroban (administered as bolus or continuous infusion) significantly reduces catheter thrombus formation equivalently to unfractionated heparin and enoxaparin.
- However, argatroban also exhibits a variable anticoagulatory efficacy depending on the mechanism of coagulation activation.
- In conclusion the data of this study encourage the use of argatroban for anticoagulation during PCI but also demonstrate a necessity for specific testing for specific anticoagulatory demands.
pharmacodynamic level by functional consumption of argatroban due to increasing thrombin generation during catheter circulation (Fig. 3): Under conditions of argatroban bolus administration a limited amount of argatroban is progressively occupied by a steadily increasing amount of thrombin that in turn results in a decreasing "thrombin inhibition capacity" as reflected by declining ACTs. Progressive thrombin generation is likely to result from continuous "contact activation" during catheter perfusion (as described below).

At first glance our present findings contradict previous data which found argatroban bolus administration to be inferior to continuous infusion in preventing thrombus formation on mechanical heart valves in vitro (31).

To focus on this discrepancy we additionally employed an alternate model of mechanical coagulation activation using a magnetic stirrer. In this experimental setup argatroban was inferior to UFH and enoxaparin in preventing appositional thrombus growth. Those findings might perhaps be explained by the qualitative and quantitative differences concerning the activation of the coagulation cascade. On artificial surfaces, thrombogenicity seems primarily to be a function of local thrombin generation rather than of platelet adhesion (32).

In a process termed "contact activation" coagulation factor XII (FXII; Hageman factor) undergoes autoactivation (FXIIa) following blood exposure to artificial surfaces (33). FXIIa then activates FXI thereby initiating a waterfall cascade that finally results in thrombin generation. Furthermore FXIIa is capable of activating the kallikrein-kinin system – which may independently induce thrombin formation (34).

However, in a setting of mechanical (shear) stress-induced coagulation, a pivotal role has been assigned to platelet activation/mechanical platelet destruction – which, in turn, potently activates and propagates the plasmatic coagulation cascade (35–37). Here, the extent of mechanical stress also determines the extent of platelet activation (37). Thus, in contrast to the low-flow conditions in our cardiac catheter model – where surface activation may be the critical event – in a scenario of mechanical heart valves as well as in our stirrer model the extent of coagulation might be dominated more by mechanical force activation that induces the coagulation cascade more extensively. This, in turn, might overload the anticoagulatory potential of DTI such as argatroban under conditions of "single shot" administration and might favour anticoagulants such as UFH and enoxaparin which inhibit the coagulation cascade at multiple sites.

Taken together, the results of this in vitro study show a significant potential of argatroban in preventing catheter thrombus formation. From that point of view, one could encourage the use of argatroban for anticoagulation during PCI. However, this study also reflects a differing efficacy of anticoagulants under varying conditions (heart valves, cardiac catheters), which demonstrates that it may not be adequate to simply extrapolate data from one study to another scenario.

Limitations

In contrast to current clinical practice, where most elective PCI procedures are performed in patients receiving established dual anti-platelet therapy (aspirin, clopidogrel), our blood donors were pretreated with aspirin only. Our study scenario, therefore, may be more analogous to PCI in the setting of ACS when active metabolites of clopidogrel are not present.

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Abbreviations

ACS, acute coronary syndromes; ACT, activated clotting time; DTI, direct thrombin inhibitor; PCI, percutaneous coronary intervention; T-AT, thrombin-antithrombin complexes; UFH, unfractionated heparin.

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