Antithrombin-independent thrombin inhibitors, but not direct factor Xa inhibitors, enhance thrombin generation in plasma through inhibition of thrombin-thrombomodulin-protein C system

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Summary
There is increasing concern that some anticoagulants can paradoxically increase thrombogenesis under certain circumstances. Previously, we demonstrated that at certain doses a direct thrombin inhibitor, melagatran, worsens the coagulation status induced by tissue factor (TF) injection in a rat model. We utilised an in vitro thrombin generation (TG) assay to define whether direct thrombin inhibitors could enhance TG in human plasma, and whether inhibition of the negative-feedback system (thrombin-thrombomodulin (TM)-protein C) contributed to the TG enhancement. TG in human plasma was assayed by means of calibrated automated thrombography. In this assay, direct factor Xa (FXa) inhibitors such as edoxaban and antithrombin (AT)-dependent anticoagulants such as heparin did not increase, but simply suppressed TG. AT-independent thrombin inhibitors (melagatran, lepirudin, and active site blocked thrombin (IIa)) increased peak levels of TG (2.0, 1.6, and 2.2-fold, respectively) in the presence of 12 nM recombinant human soluble TM (rhsTM). Melagatran and lepirudin at higher concentrations began to suppress TG. In the absence of rhsTM, the enhancement of peak TG by melagatran decreased to 1.2-fold. Furthermore, in protein C-deficient plasma, AT-independent thrombin inhibitors failed to enhance TG. In addition, a human protein C neutralising antibody increased the peak height of TG in the presence of rhsTM. These results suggest that AT-independent thrombin inhibitors may activate thrombogenesis by suppression of the thrombin-induced negative-feedback system through inhibition of protein C activation. In contrast, direct FXa inhibitors are more useful than AT-independent thrombin inhibitors in terms of lower possibility of activation of the coagulation pathway.

Keywords
Thrombin inhibitor, factor Xa inhibitor, protein C, thrombomodulin, negative-feedback system

Introduction
Recurrent thrombotic events upon cessation of anticoagulant therapies are a crucial issue in antithrombotic therapy. Clinical studies show that withdrawal of heparin is associated with rebound coagulation activation in patients with coronary artery disease (1, 2). In addition, ximelagatran, a prodrug of direct thrombin inhibitor (DTI) melagatran, significantly increases risk of arterial cardiovascular events compared with warfarin/placebo in studies for the prevention of venous thromboembolism (3). Recently, the Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) study (4) demonstrated that another direct thrombin inhibitor, dabigatran, very slightly increases the rate of myocardial infarction compared to warfarin (0.74%/year vs. 0.53%/year) while the compound lowered the rates of stroke and systemic embolism more obviously (1.11%/year vs. 1.69%/year).

We have previously demonstrated that melagatran inhibits and activates coagulation in rats (5). At certain doses, melagatran increases hypercoagulability in a rat model of tissue factor (TF)-induced hypercoagulation. This paradoxical phenomenon may be implicated in rebound coagulation activation observed with thrombin inhibitors in clinical trials. On the other hand, a factor Xa (FXa) inhibitor, DX-9065a, did not exert any deleterious effects on a rat model of TF-induced hypercoagulation.

Thrombin and FXa play a central role in the blood coagulation cascade. Thrombin is a multifunctional trypsin-like serine protease that converts fibrinogen to fibrin (6). In addition, thrombin induces platelet activation and triggers a wide range of effects, including vascular smooth muscle cell and fibroblast proliferation, monocyte chemotaxis, and neutrophil adhesion. Furthermore, thrombin binds to thrombomodulin (TM) and negatively regulates the coagulation system. Compared to thrombin, FXa has limited function, serving as the principal mediator of thrombin generation (TG) from prothrombin by the prothrombinase complex (7).

Protein C is an important factor for negative regulation of the coagulation pathway (8, 9). Thrombin-TM complex activates pro-
tein C, and the activated protein C exerts a potent anticoagulant effect via proteolysis of factor Va and VIIIa. Melagatran inhibits the activation of protein C through the inhibition of TM-bound thrombin (10). We hypothesise that protein C is one of the factors that contributes to the hypercoagulation observed by thrombin inhibitors in the rat model. In contrast, it is unlikely that direct FXa inhibitors would inhibit protein C activation directly, because they are highly selective for FXa (11, 12).

To determine the mechanism of the thrombin inhibitor-induced paradoxical coagulation activation, we utilised an in vitro TG assay. TG assay provides useful information about thrombotic (13–15) or haemorrhagic risk (16, 17). Calibrated Automated Thrombogram (CAT) has become available to assess in vitro TG in plasma after activation of coagulation with TF, phospholipids, and CaCl₂ (18, 19).

In the present study, we determined whether antithrombin (AT)-independent, -dependent thrombin inhibitors and direct FXa inhibitors enhance TG in human plasma, and this enhancement is mediated by inhibition of the thrombin-TM and protein C system.

Figure 1: Effects of AT-independent thrombin inhibitors on TF-induced TG in human plasma in the presence of TM. Melagatran (A), lepirudin (B), and IIai (C) increased TG in the presence of TM. Increase in the peak height of TG reached maximal at 300 nM of melagatran (2.4-fold), 13 nM of lepirudin (1.6-fold), and 608 nM of IIai (2.2-fold). At higher concentrations, melagatran and lepirudin suppressed the peak level of TG. All experiments except for lepirudin (n=1) were performed four times. The typical data are presented.

Figure 2: Effects of direct factor Xa inhibitors on TF-induced TG in human plasma in the presence of TM. Edoxaban (A) and DX-9065a (B) decreased TG in concentration-dependent manners. All experiments were performed four times. The typical data are presented.
Materials and methods

Reagents and drugs

Edoxaban tosylate, DX-9065a, and melagatran were synthesised and a phospholipid reagent (60% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylserine) and IIai were prepared at Daiichi Sankyo (formerly Daiichi Pharmaceutical, Tokyo, Japan). Heparin (Mochida Pharmaceutical, Tokyo, Japan), dalteparin (Kissei Pharmaceutical, Nagano, Japan), and fondaparinux (Sanofi-Aventis, Paris, France) were purchased. Normal and protein C-deficient human plasma were purchased from George King Bio-Medical, Inc. (Overland Park, KS, USA). Fluorogenic thrombin substrate Z-Gly-Gly-Arg-aminomethylcoumarin (Z-GGR-AMC) was bought from Bachem AG (Bubendorf, Switzerland). Polyclonal rabbit anti-human protein C antibody was from Dako Cytomation (Carpinteria, CA, USA) and recombinant human soluble thrombomodulin (rhsTM) was from American Diagnostica (Stamford, CT, USA). Platelin LS was from by bioMérieux Japan (Tokyo, Japan) and human activated protein C was from Sigma-Aldrich (St. Louis, MO, USA). The thrombin calibrator and PPP-Reagents were from Thrombinoscope BV (Maastricht, The Netherlands).

TG assay

TG in platelet-poor plasma was assayed by means of the CAT with a fluorometer Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA) and the thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) (18). Briefly, the assay was performed as follows, 80 μl plasma in the presence and absence of anticoagulants, rhsTM, and protein C were pipetted into the well of a microtiter plate together with 20 μl of a mixture of 15 pM TF and 24 μM phospholipids (diluted PPP reagent). After 5 minutes (min) of preincubation at 37°C, the reaction was started with 20 μl of a mixture of 2.5 mM fluorogenic substrate (Z-GGR-AMC) and 100 mM CaCl₂. Final concentrations were 2.5 pM TF, 4 μM phospholipids, 16.7 mM CaCl₂, and 417 μM fluorogenic substrate.

To determine the role of protein C, purified activated protein C or anti-human protein C antibody was added to normal plasma or PC-deficient plasma.

The fluorescence was measured for 60 min at 37°C (ex. 390 nm, em. 460 nm). TG curves were described in terms of peak height, lag time, and endogenous thrombin potential (ETP).

Activated partial thromboplastin time (aPTT) assay

aPTT was measured using a microcoagulometer, Amelung KC-10A (Trinity Biotech Plc., Bray, Ireland), by incubating 50 μl plasma with anti-human protein C antibody or rabbit IgG, 50 μl...
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Figure 4: Effects of AT-independent thrombin and FXa inhibitors on the parameters of TG in human plasma in the presence of TM. AT-independent thrombin inhibitors increased the peak height (A). Melagatran and lepirudin prolonged lag time (C) in a concentration-dependent manner, whereas Ilai did not affect it. AT-independent FXa inhibitors exerted suppression of the peak height (B) and prolongation of lag time (D). Data represent means ± SEM (n=4) except for lepirudin (n=1). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control (Dunnett multiple comparison test).

Statistical analysis
Analyses were performed using EXSAS ver.7.10 (ARM SYTEX, Osaka, Japan) based on SAS release 8.2 (SAS Institute Japan, Tokyo, Japan). The data (the peak height and lag time of TG, and aPTT) represent means ± standard errors of mean (SEM) and statistical significance was measured at the level of p < 0.05. The statistical significance of the data was analysed by Bartlett and Dunnett multiple comparison tests.

Results
Effects of anticoagulants on TG in the presence of TM

TG was determined utilising human plasma in the presence of 12 nM rhsTM.

(1) AT-independent thrombin inhibitors
Melagatran (a direct thrombin inhibitor), lepirudin (recombinant hirudin), and Ilai (active site blocked thrombin) increased TG in the presence of rhsTM. Maximum peak height of TG was reached at 300 nM of melagatran (2.0-fold), 13 nM of lepirudin (1.6-fold), and 608 nM of Ilai (2.2-fold) (Figs. 1, 4A). At higher concentrations, melagatran and lepirudin suppressed the peak height of TG. These inhibitors also increased ETP (data not shown). In terms of the time parameter, melagatran and lepirudin prolonged lag time with increasing concentrations, whereas Ilai did not have an effect (Fig. 4C).

(2) Direct FXa inhibitors
Edoxaban and DX-9065a concentration-dependently decreased TG (Figs. 2, 4B and D). Both FXa inhibitors suppressed the peak height and ETP and prolonged lag time (Figs. 2, 4B and D).

(3) AT-dependent anticoagulants
Heparin, dalteparin, and fondaparinux inhibited TG (Fig. 3). Similar to direct FXa inhibitors, no enhancement was detected. These anticoagulants did not prolong lag time.

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rhsTM, however, TG was more obviously facilitated by 150 nM melagatran. The effect of rhsTM was concentration-dependent; at 12 nM the peak height of TG by melagatran (150 nM) increased 2.2-fold (Fig. 5B). This finding suggests the significant role of the negative-feedback system via TM in this aggravation of TG by the thrombin inhibitors.

Direct FXa inhibitors (edoxaban and DX-9065a) and AT-dependent anticoagulants (heparin, dalteparin, and fondaparinux) exerted inhibitory effects both in the absence and presence of rhsTM (data not shown).

(2) Role of protein C
The effects of thrombin inhibitors on TG were examined in protein C-deficient human plasma. Melagatran, lepirudin, and IIai failed to enhance TG in protein C-deficient plasma, even in the presence of 12 nM rhsTM (Fig. 6). Direct FXa inhibitors and AT-dependent anticoagulants also did not enhance TG in protein C-deficient plasma (data not shown).

Then, we investigated the effect of anti-human protein C neutralising antibody. Neutralisation of protein C by the antibody was
confirmed by measuring aPTT of human plasma (Fig. 7A). The protein C activator, Protac, prolonged aPTT. Anti-human protein C neutralising antibody concentration-dependently shortened aPTT prolonged by Protac (A). Anti-human protein C neutralising antibody (0.1 – 0.4 μM) increased the peak height of TG in the presence of TM (B, C). Data represent means ± SEM (n=4). *** p < 0.001 vs. control (Dunnett multiple comparison test).

Furthermore, we studied the effect of activated protein C on increased TG by DTI. Activated protein C concentration-dependently reversed enhanced peak TG by 150 nM melagatran in TM-added normal plasma (Fig. 8).

These results suggest that protein C plays a critical role in the potentiation of TG by AT-independent thrombin inhibitors.

**Discussion**

We have previously demonstrated that melagatran aggravates coagulation status in TF-induced hypercoagulation rats (5). In contrast, direct FXa inhibitor, DX-9065a, did not show any exacerbation (5). In this study, we determined the effects of melagatran on TG in human plasma in vitro to mimic the paradoxical activation of coagulation. Then, we compared the effects of various anticoagulants on TG. Moreover, to clarify the precise mechanism of the enhancement of coagulation, we examined whether the negative feedback system by TM-protein C is included in the enhancement of TG by AT-independent thrombin inhibitors.

We measured TG in human plasma containing rhsTM to examine the coagulation response under conditions where the negative-feedback system is active as in an in vivo situation. The extrapolation of in vitro findings to the in vivo situation is not easy, because TM is a membrane protein expressed on the surface of endothelial cells. TM concentration is estimated from < 1 nM (vessels with a diameter > 0.5 mm) to above 100 nM in the capillaries (20). Therefore, the concentration of sTM that used in our study is appropriate. Melagatran at concentrations of 75–600 nM (4–315 ng/ml) enhanced TG in the presence of rhsTM (Fig. 7B, C). Control antibody had no effect on TG.

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Figure 9: A possible mechanism underlying AT-independent thrombin inhibitors enhance TG. Based on our observations and the reports from other laboratories, the possible mechanism underlying AT-independent thrombin inhibitors enhance TG is summarised above. Phospholipids (PL) and Ca$^{2+}$ are necessary, but not shown in the figure.

The patterns of effects on TG by melagatran, lepirudin and IIai differ from one another. The enhancement of the peak height and ETP by melagatran is more pronounced than lepirudin. Interestingly, IIai did not affect the lag time. These differences are probably due to their mechanism of action. Melagatran inhibits both free and TM-bound thrombin and therefore also inhibits the generation of activated protein C (21). Lepirudin is a selective thrombin inhibitor. These differences in mechanism of action from AT-independent thrombin inhibitors may contribute to differential results. Further studies may be required to clarify the distinctions.

Direct FXa inhibitors like edoxaban also did not enhance TG in human plasma in the absence and presence of TM and protein C. Furthermore, edoxaban did not show any exacerbation regardless of its plasma concentration in TF-induced hypercoagulation rats (in preparation). The effects of direct thrombin inhibitors depend on their plasma concentration and their activities decreased at trough levels, whereas the activity of warfarin is consistent for 24
hours. Therefore, we speculate that when the concentrations of direct thrombin inhibitors decline below therapeutic ranges, the paradoxical coagulation phenomenon in patients with coronary artery diseases.

We have shown that low-dose administration of a direct thrombin inhibitor, melagatran, significantly worsens the coagulation status and a direct factor Xa inhibitor, DX-9065a, did not exert any deleterious effects in a rat model of tissue-factor-induced hypercoagulation.

The precise mechanisms of the paradoxical coagulation activation by melagatran are unclear.

What does this paper add?

- Antithrombin (AT)-independent thrombin inhibitors (melagatran, lepirudin, and active site blocked thrombin) increased thrombin generation (TG) in the presence of soluble thrombomodulin.
- This increasing of TG was not observed in protein C-deficient plasma.
- The mechanisms of the paradoxical coagulation activation by melagatran may be suppression of the thrombin-induced negative-feedback system through inhibition of protein C activation.
- Direct factor Xa inhibitors and AT-dependent anticoagulants did not increase TG.

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Conflict of interests

All authors are employees of Daiichi Sankyo Co., Ltd.

References