Effect of myeloperoxidase on the anticoagulant activity of low molecular weight heparin and rivaroxaban in an in vitro tumor model

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Abstract

Background: Inflammation with leukocyte activation is a hallmark of cancer-associated thrombosis (CAT), and elevated leukocytes predict venous thromboembolism in cancer outpatients. In a recent trial, rivaroxaban was more efficacious than dalteparin in preventing CAT recurrence.

Objectives: In a proof-of-concept study, we aimed to provide a mechanistic basis for improved efficacy of rivaroxaban compared to low molecular weight heparin in CAT treatment.

Methods: We studied the effects of rivaroxaban, dalteparin, and tinzaparin at peak and trough levels on tumor cell–induced procoagulant activity and platelet aggregation in the presence or absence of the cationic leukocyte-derived enzyme, myeloperoxidase (MPO). Furthermore, pro-inflammatory conditions were generated by stimulating whole blood with lipopolysaccharide (LPS) or phorbol-myristate-acetate (PMA), before measuring thrombin generation in plasma supernatants.

Results: All three anticoagulants inhibited thrombin generation, fibrin clot formation, and platelet aggregation induced by the tissue factor-expressing prostate carcinoma cell line, 22Rv1. Pre-incubation with MPO partially attenuated the anticoagulant activity of dalteparin and tinzaparin, but not rivaroxaban, at trough levels. The effect of MPO did not involve the enzyme’s catalytic properties, but required its structural integrity, as indicated by heat denaturation. In plasma obtained from LPS- or PMA-stimulated whole blood, elevated MPO antigen levels inversely correlated with the ability of tinzaparin to inhibit 22Rv1-induced thrombin generation.

Conclusions: Myeloperoxidase release may partially attenuate the anticoagulant activity of trough levels of dalteparin and tinzaparin in the context of paraneoplastic leukocyte activation. However, this effect is likely not sufficient to explain the...
1 | INTRODUCTION

Cancer is associated with a four- to seven-fold increased risk of venous thromboembolism (VTE), which constitutes a major healthcare burden with significant impact on the quality of life of affected patients.\(^1\) Thromboembolic events are a leading cause of death in cancer outpatients,\(^2\) and cancer patients with VTE have a less favorable clinical outcome than cancer patients without VTE.\(^3\) About two thirds of patients with cancer-associated VTE die within one year after its diagnosis.\(^4\) The occurrence of VTE may not only reflect a particularly aggressive and/or advanced malignancy, but may also delay effective anticancer treatment.

Inflammation is a hallmark of cancer-associated VTE, and elevated leukocytes, particularly neutrophils, are predictive of VTE in cancer patients receiving ambulatory chemotherapy.\(^5,6\) Neutrophils may trigger thrombus formation through the exposure of tissue factor (TF), cytokine production, and release of proteases.\(^7,8\) Recently, neutrophil extracellular traps (NETs), comprising cell-free DNA bound to granular and cytoplasmic antimicrobial proteins, have been shown to significantly contribute to cancer-associated thrombosis.\(^9,10\) Most importantly, NETs promote platelet trapping and activation, while activated platelets, in turn, stimulate NETosis.\(^11,12\) Moreover, accumulation of erythrocytes and release of von Willebrand factor are mechanisms by which NETs trigger thrombus formation.\(^13\)

Myeloperoxidase (MPO) is an abundant protein in neutrophils and an essential constituent of NETs.\(^14,15\) As a heme peroxidase it catalyzes the formation of microbicidal hypochlorous acid. Interestingly, MPO also has immunomodulatory effects that are independent of its catalytic activity.\(^16\) In this regard, electrostatic interactions between the highly cationic enzyme and negatively charged structures likely play a role.\(^17\) Elevated levels of MPO in malignant tissues and increased MPO serum levels in patients with breast cancer are consistent with a triangular network formed by bidirectional relationships between cancer, inflammation, and thrombosis.\(^18-21\)

For almost two decades, parenteral anticoagulation with low molecular weight heparin (LMWH) has been the treatment of choice for cancer-associated VTE.\(^2\) In this regard, two pivotal clinical trials, CLOT and CATCH, have indicated that dalteparin and tinzaparin are superior to vitamin K antagonists with regard to relevant safety and efficacy endpoints.\(^22-24\) Recently, direct oral anticoagulants (DOACs) have also been evaluated in this setting. In the prospective, randomized pilot study, SELECT-D, the factor Xa inhibitor rivaroxaban (15 mg BID for 3 weeks, followed by 20 mg OD) was compared to dalteparin (200 IU/kg OD for 30 days, followed by 150 IU/kg OD) in 406 patients with active cancer and newly diagnosed VTE.\(^25\) Over the 6-month treatment period, rivaroxaban (versus dalteparin) was associated with a significant reduction in recurrent VTE (4% versus 11%; hazard ratio [HR] 0.43, 95% confidence interval [CI] 0.19-0.99), a finding that could not be explained by improved treatment persistence in the DOAC arm. While there was no difference in major bleeding, the cumulative incidence of clinically relevant non-major bleeding was significantly higher in the rivaroxaban than in the dalteparin group (13% versus 4%; HR 3.76, 95% CI 1.63-8.69).

In contrast to LMWH, rivaroxaban inhibits factor Xa independently of antithrombin.\(^26\) In addition, rivaroxaban inhibits both free and clot-bound factor Xa. However, the mechanistic basis for improved efficacy of rivaroxaban compared to LMWH in patients with cancer-associated VTE remains obscure.

In this proof-of-concept study, we hypothesized that leukocyte-derived MPO may interfere with the anticoagulant activity of LMWH in an in vitro model of tumor cell-induced thrombosis.

2 | METHODS

2.1 | Materials

The following commercially available reagents were used: tinzaparin, dalteparin, lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4), phorbol 12-myristate 13-acetate (PMA), thrombin receptor-activating peptide-6 (TRAP-6, SFLRNR), human serum albumin (HSA), apyrase (all from Sigma Aldrich), rivaroxaban (Santa Cruz Biotechnology), normal human plasma (NHP; HemosIL®; Instrumentation Laboratory), lipidad recombinant human full-length tissue factor (rhTF; Innovin®, Siemens Healthcare), inhibitory TF monoclonal antibody no. 4509 (anti-TF; Sekisui

improved efficacy of rivaroxaban, and possibly other oral factor Xa inhibitors, in CAT treatment.

**KEYWORDS**

low molecular weight heparin, myeloperoxidase, prostate cancer, rivaroxaban, thrombosis

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**Essentials**

- Cancer-associated thrombosis (CAT) is characterized by inflammation with leukocyte activation.
- Rivaroxaban was compared with low molecular weight heparin (LMWH) in an in vitro CAT model.
- At trough levels of anticoagulants, MPO partially inhibited LMWH, but not rivaroxaban.
- Release of MPO by leukocytes cannot solely explain the reduced efficacy of LMWH in CAT trials.
Diagnostics), MPO isolated from polymorphonuclear leukocytes (PMNs; Planta Natural Products), thrombin (Siemens, Berlin and Munich).

2.2 Cell line and culture

The human prostate carcinoma 22Rv1 cell line was obtained from ATCC and maintained in RPMI culture medium supplemented with 10% heat-inactivated fetal calf serum at 37°C and 5% CO₂.

2.3 Thrombin generation assay

22Rv1-induced thrombin generation was recorded with a modified fluorogenic thrombin generation assay (TGA; Technoclone) on a BioTek® FLx800 TBI fluorometer. Briefly, commercially supplied NHP was depleted from its endogenous procoagulant phospholipid (PL) vesicles by double high-speed centrifugation at 16 100 x g for 30 minutes in an Eppendorf microcentrifuge to obtain PL-free plasma (PLFP). Harvested and washed 22Rv1 cells were resuspended in phosphate-buffered saline (PBS) at various concentrations and then diluted 1:10 with PLFP. Forty microliters of each sample were transferred into duplicate wells of a 96-well microtiter plate. Following addition of 60 µL substrate solution containing 1 mmol/L Z-G-G-R-AMC fluorogenic peptide and 15 mmol/L CaCl₂, thrombin generation was recorded for up to 90 minutes. To demonstrate TF concentrations were as follows: 22Rv1 cells 2 x 10⁵, 28,29,31 tinzaparin (0.2 IU/mL), rivaroxaban 26 µg/L, MPO 20 µg/mL.

2.4 Single-stage clotting assay

22Rv1-induced fibrin clot formation in PLFP was measured using a KC10 coagulation instrument (Amelung). One hundred µl of cell suspension was incubated with 100 µl of PLFP for 2 minutes at 37°C. Following the addition of 50 µl CaCl₂ (25 mmol/L), times until fibrin clot formation were recorded and converted into arbitrary units (AU) by reference to a standard curve obtained by serial dilutions of rhTF. All assays were performed in duplicate.

2.5 Inhibition of 22Rv1-dependent PCA by LMWH and rivaroxaban

To investigate the effect of dalteparin, tinzaparin, and rivaroxaban on 22Rv1-induced procoagulant activity (PCA), peak or trough concentrations of the respective anticoagulants were introduced into the thrombin generation and fibrin clot formation assay. Final concentrations were as follows: 22Rv1 cells 2 x 10⁷/mL, tinzaparin (dosed at 175 IU/kg OD) 0.85 or 0.2 IU/mL, 28-30 dalteparin (200 IU/kg) 1.05 or 0.2 IU/mL, 28,29,31 rivaroxaban (20 mg OD) 270 or 26 µg/L. 32

2.6 Effect of MPO on the anticoagulant activity of LMWH and rivaroxaban

In order to evaluate the effect of MPO on the anticoagulant activity of LMWH and rivaroxaban, each anticoagulant was pre-incubated with PBS or HSA (controls) or MPO for 30 minutes at 37°C before the PCA of 22Rv1 cells was measured by TGA or fibrin clot formation assay, as described above. In typical experiments, final concentrations were as follows: 22Rv1 cells 2 x 10⁷/mL, tinzaparin and dalteparin 0.2 IU/mL, rivaroxaban 26 µg/L, MPO 20 µg/mL.

2.7 Induction of MPO release in whole blood and measurement of thrombin generation in plasma supernatants

Citrate-anticoagulated whole blood from healthy volunteers was treated with PBS, 10 µg/mL LPS or 10 µmol/L PMA for 5 hours at 37°C and subsequently double centrifuged (2 x 10 minutes at 2060 x g) to obtain platelet-poor plasma (PPP). PLFP was prepared from PPP as described above and stored at -80°C. 22Rv1-induced thrombin generation was measured in thawed PLFP in the presence or absence of tinzaparin (0.2, 0.1, and 0.05 IU/mL) or rivaroxaban (26 µg/L). In addition, MPO antigen was quantified in PLFP using the Human Myeloperoxidase Enzyme Immunoassay Kit (Arbor Assays) according to the manufacturer’s instructions.

2.8 22Rv1-triggered platelet aggregation

Citrate-anticoagulated whole blood was centrifuged for 15 minutes at 120 x g to obtain platelet-rich plasma (PRP). Following the addition of 2 U/mL apyrase, platelets were pelleted by centrifugation for 10 minutes at 580 x g and resuspended in modified Tyrode’s buffer (25 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 3.3 mmol/L NaH₂PO₄, 5.55 mmol/L glucose, and 20 mmol/L HEPES, pH 7.4) to a final concentration of 150 x 10⁵/µL. Platelet suspensions were supplemented with NHP (1:100, vol:vol) and spiked with PBS (control), tinzaparin (0.2 IU/mL), or rivaroxaban (26 µg/L). When indicated, anticoagulants were pre-incubated with MPO (20 µg/mL) for 30 minutes at 37°C. 22Rv1 cells were harvested, washed, and resuspended in modified Tyrode’s buffer containing 20 mmol/L CaCl₂. Aggregation was initiated by adding 25 µL TRAP-6 (1 mmol/L), thrombin (1 IU/mL), or 22Rv1 cells (2 x 10⁷/mL) to 225 µL platelet suspension in an APACT™ light transmittance aggregometer (Rolf Greiner BioChemica). Modified Tyrode’s buffer containing CaCl₂ served as negative control.

2.9 Statistical analysis

All experiments were repeated at least three times. Normally distributed data were presented as mean ± standard deviation (SD) and analyzed by two-sided Student’s t-test for paired observations.
FIGURE 1  Inhibition of 22Rv1-induced procoagulant activity by tinzaparin, dalteparin, and rivaroxaban. Antithrombotic effects of peak (A, C, E, G) and trough (B, D, F, H) levels of dalteparin (1.05 and 0.2 IU/mL), tinzaparin (0.85 and 0.2 IU/mL), and rivaroxaban (270 and 26 µg/L) on peak thrombin (A, B), the area under the thrombin generation curve (AUC) (C, D), lag time (E, F), and fibrin clot formation (G, H) triggered by 22Rv1 cells (2 × 10^5/mL), using a modified fluorogenic thrombin generation and single-stage clotting assay, respectively (n = 6). Multiple comparisons were calculated by analysis of variance. *P < .05, **P < .01, ***P < .001.
Differences between multiple approaches were studied by analysis of variance and Tukey's post hoc test. Correlation coefficients were according to the method of Pearson. Analyses were carried out using IBM SPSS version 22 (IBM SPSS). A P value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | PCA of 22Rv1 cells is TF-dependent

We used a modified thrombin generation and single-stage clotting assay to measure the PCA of 22Rv1 cells. While essentially no PCA was detectable in the absence of tumor cells in both assays, addition of 22Rv1 cells induced thrombin generation and fibrin clot formation in a concentration-dependent manner (Figure S1 in supporting information). 22Rv1-induced thrombin generation was TF-dependent as demonstrated by pre-incubation with an inhibitory TF monoclonal antibody (anti-TF).

3.2 | Rivaroxaban inhibits 22Rv1-induced thrombin generation less efficaciously than tinzaparin and dalteparin

At peak anti-Xa concentrations, both tinzaparin and dalteparin abolished thrombin generation induced by 22Rv1 cells (2 x 10⁵/mL), while at trough anti-Xa concentrations, peak thrombin (PT) and the area under the thrombin generation curve (AUC) were reduced by 70% to 80% (Figure 1A-D, Figure S2 in supporting information). Compared to both LMWHs, rivaroxaban was significantly less efficacious in inhibiting 22Rv1-induced thrombin generation, with residual PT and AUC levels of 17% and 49%, respectively, at peak concentrations and 69% and 92%, respectively, at trough concentrations (P < .001 for each comparison between LMWH and rivaroxaban). All three anticoagulants increased the lag time by about three-fold at peak concentrations and by about 1.5-fold at trough concentrations (Figure 1E,F). Similar findings were obtained when PCA was assessed by single-stage clotting assay: compared to dalteparin and tinzaparin, rivaroxaban was less efficacious in inhibiting fibrin clot formation at both peak (Figure 1G) and trough (Figure 1H) concentrations (P < .001 for each comparison).

3.3 | Exogenous MPO partially reverses the anticoagulant activity of tinzaparin in a dose-dependent manner

Next, we investigated the effect of exogenous MPO on the inhibitory activity of tinzaparin on 22Rv1-induced thrombin generation and fibrin clot formation. To this end, increasing concentrations of MPO (1-20 µg/mL) were pre-incubated with trough levels of the anticoagulant (Figure 2A). While MPO at 1 µg/mL had no effect, the anticoagulant's inhibitory activity on 22Rv1-induced thrombin generation was partially reversed at higher MPO concentrations. At 20 µg/mL, MPO significantly increased residual PT levels from 12% to 34% and residual AUC levels from 11% to 32% (Figure 2B,C). Similarly, the anticoagulant activity of tinzaparin on 22Rv1-induced fibrin clot formation was significantly attenuated by 20 µg/mL MPO (Figure 2D). To demonstrate MPO specificity, tinzaparin was treated with HSA at concentrations up to 100 µg/mL, which had no effect on the LMWH's anticoagulant activity (Figure S3A in supporting information). In addition, MPO-mediated inhibition of the anticoagulant activity of tinzaparin could not be further increased by adding 80 µmol/L H₂O₂ (Figure S3B), indicating that the enzyme's catalytic properties were dispensable. In contrast, heat denaturation at 95°C reversed the effect of MPO on inhibition of 22Rv1-induced thrombin generation by tinzaparin, while MPO heating to 37°C or 56°C did not (Figure S3C). We therefore conclude that the effect of MPO on the anticoagulant activity of tinzaparin is not dependent on the enzyme's catalytic activity, but may involve electrostatic interactions between the cationic MPO and the negatively charged glycosaminoglycan.

3.4 | Exogenous MPO attenuates the anticoagulant activity of LMWH, but not rivaroxaban

We also investigated the effect of MPO (20 µg/mL) on trough concentrations of dalteparin and rivaroxaban. While MPO (versus no MPO) also mitigated the anticoagulant activity of dalteparin on 22Rv1-induced thrombin generation, as indicated by residual levels of PT (61% versus 27%, P < .001) and AUC (57% versus 25%, P < .001), MPO had no significant effect on the anticoagulant activity of rivaroxaban (Figure 3A,B). Similar results were obtained when PCA was analyzed by single-stage clotting assay (Figure 3C).

3.5 | Endogenously released MPO inversely correlates with the anticoagulant activity of tinzaparin in LPS- or PMA-stimulated whole blood

In a subsequent set of experiments, we investigated the anticoagulant activity of tinzaparin under pro-inflammatory conditions. To this end, citrate-anticoagulated whole blood was stimulated with LPS (10 µg/mL) or PMA (10 µmol/L) for 5 hours at 37°C, and 22Rv1-induced thrombin generation was subsequently measured in plasma supernatants in the presence or absence of the anticoagulant (0.05-0.2 IU/mL). Prior to the TGA, plasma supernatants were centrifuged twice at 16 100 × g for 30 minutes to remove endogenous (TF-bearing) microvesicles generated during the stimulation process. Whole blood incubated with buffer (PBS) served as negative control.

In both PBS- and LPS- or PMA-treated samples, tinzaparin attenuated 22Rv1-triggered thrombin generation, expressed as AUC, in a
concentration-dependent manner (Figure 4A). However, the anticoagulant activity of tinzaparin at any concentration was significantly reduced in PMA- as compared to PBS-treated samples, while the difference in 22Rv1-triggered thrombin generation between PBS- and...
LPS-treated samples was only statistically significant at 0.05 and 0.1 IU/mL tinzaparin. The anticoagulant activity of tinzaparin was consistently less potent in PMA- as compared to LPS-treated samples. Similar findings were obtained when thrombin generation was expressed as PT (Figure 4B).

Compared to plasmas obtained from PBS-treated whole blood (7.7 ± 1.9 ng/mL), MPO antigen levels, as measured by enzyme-linked immunosorbent assay (ELISA), were significantly increased in LPS-treated (208.8 ± 29.5 ng/mL) or PMA-treated (393.1 ± 17.5 ng/mL) samples (Figure 4C). Stimulation of whole blood with PMA was more efficacious than stimulation with LPS in inducing MPO release (P < .001). MPO antigen levels in plasma supernatants from PBS-, LPS-, or PMA-treated whole blood significantly correlated with residual thrombin generation in the presence of tinzaparin (Figure 4D). Of note, even within LPS-treated samples (n = 4), there was a close inverse correlation between MPO antigen levels and the ability of tinzaparin to inhibit 22Rv1-induced thrombin generation (Figure S4 in supporting information).

Consistent with our previous findings (Figure 1), trough concentrations of rivaroxaban inhibited 22Rv1-induced thrombin generation in PBS-treated samples, albeit to a much lesser extent than the LMWH (Figure S5A, B in supporting information). Despite this apparently reduced anticoagulant activity of rivaroxaban compared to tinzaparin, 22Rv1-induced thrombin generation in the presence of 26 µg/L rivaroxaban was not further increased in LPS- or PMA-treated samples.

Collectively, these findings indicate that attenuation of tinzaparin's anticoagulant activity under artificially induced pro-inflammatory conditions is correlated with the release of MPO from PMNs and monocytes.

3.6 | MPO interferes with the inhibitory effect of tinzaparin, but not rivaroxaban on 22Rv1-triggered platelet aggregation

Finally, we investigated the effect of tinzaparin and rivaroxaban on 22Rv1-induced platelet aggregation and its modulation by MPO. By light transmittance aggregometry (LTA) no aggregation of washed platelets resuspended in buffer containing 1:100 (vol:vol) plasma was detected upon addition of 2 mmol/L CaCl$_2$ (Figure S6A in supporting information). The protease-activated receptor-1 (PAR-1) agonist peptide SFLLRN (TRAP-6) induced immediate, monophasic platelet aggregation. Platelet aggregation triggered by 22Rv1 cells (2 × 10$^4$/mL), however, resembled the thrombin-induced response, showing a biphasic aggregation curve, which is likely explained by an initial thrombin-mediated effect on platelet activation and a more delayed thrombin-mediated effect on fibrin strand formation, contributing to platelet clumping. 22Rv1-triggered platelet aggregation was TF-dependent (Figure S6B). Addition of tinzaparin at trough concentrations (0.2 IU/mL) inhibited 22Rv1-induced platelet aggregation, and this effect was attenuated by pre-incubation with MPO (Figure 5A). In contrast, MPO did not affect the inhibitory effect of rivaroxaban on 22Rv1-induced platelet aggregation (Figure 5B).

4 | DISCUSSION

In this proof-of-concept study, we investigated the effect of leukocyte-derived MPO on the anticoagulant activity of the direct oral factor Xa inhibitor, rivaroxaban, and two LMWHs, tinzaparin and dalteparin, in an in vitro model of tumor cell-induced thrombosis. Our findings indicate that MPO specifically interferes with the anticoagulant activity of both LMWHs, which could at least theoretically provide a mechanistic basis for recent clinical trial findings.
We have chosen a prostate cancer (PC) cell line that promoted robust TF-dependent thrombin generation, fibrin clot formation, and platelet aggregation. In clinical practice, PC is associated with a comparatively low incidence of VTE. However, the risk of VTE in PC is strongly correlated with tumor spread, with advanced stages conferring a significantly higher risk of VTE than localized stages. In addition, PC is the most frequent cancer in men, and PC-associated VTE thus represents a major health-care burden.

Interestingly, MPO is present at the surface of epithelial cells in normal prostate tissue, benign prostatic hyperplasia, and PC, suggesting a potential role of MPO in the pro-inflammatory pathways leading to (pre-)malignant prostate lesions. Finally, TF-driven systemic coagulopathies such as disseminated intravascular coagulation (DIC) may complicate the clinical management of patients with advanced PC, further indicating that this tumor entity has profound effects on the clotting system. Because TF has been implicated in the pathogenesis of VTE in many other solid tumor entities, including pancreatic, lung, and ovarian cancer, we believe that our findings using the PC cell line 22Rv1 may be generalizable to other clinical scenarios of cancer-associated thrombosis (CAT).

At both peak and trough concentrations, rivaroxaban was significantly less efficacious than the two LMWHs in inhibiting thrombin generation and fibrin clot formation (Figure 1A-D). In addition, trough levels of rivaroxaban inhibited 22Rv1-induced platelet aggregation less potently than 0.2 IU/mL tinzaparin (Figure 5). At this point it is important to clarify that, despite having a different

**FIGURE 4** Endogenously released myeloperoxidase (MPO) inversely correlates with the anticoagulant activity of tinzaparin on 22Rv1-induced thrombin generation under inflammatory conditions. A, B, Following incubation of whole blood with buffer (phosphate-buffered saline [PBS]), lipopolysaccharide (LPS) 10 µg/mL or phorbol-myristate-acetate (PMA) 10 µM for 5 hours at 37°C, antithrombotic effects of tinzaparin (0.05, 0.1, and 0.2 IU/mL) on 22Rv1-induced thrombin generation in double high-speed centrifuged plasma supernatants (phospholipid-free plasma [PLFP]) were assessed by modified thrombin generation assay (TGA). Results were expressed as area under the curve (A) or peak thrombin (B) (n = 4). Multiple comparisons were calculated by analysis of variance. P values are according to Tukey’s post hoc test. C, MPO antigen levels were measured in plasma supernatants by enzyme-linked immunosorbent assay. P values are according to Student’s t-test. D, MPO antigen levels were plotted against residual 22Rv1-induced thrombin generation in plasma supernatants from PBS-, LPS-, or PMA-treated whole blood samples in the presence of various concentrations of tinzaparin. Correlation coefficients (r) are according to the method of Pearson. ***P < .001, **P < .01, *P < .05
readout, all three test systems used in our study depend on the same mechanism, that is TF-driven thrombin generation, and the apparently inferior efficacy of rivaroxaban was particularly evident in the modified TGA. However, caution is warranted when interpreting this finding. Tinzaparin and dalteparin are comparatively long-chained LMWHs with an average molecular weight of approximately 6 kDa as compared to, for instance, enoxaparin with an average molecular weight of 4.5 kDa. In addition to inhibiting factor Xa, dalteparin and tinzaparin have significant anti-factor Ila (anti-thrombin) activity, which probably explains the profound inhibition of 22Rv1-induced thrombin generation even at trough levels. For this reason, the absolute magnitude of anticoagulant activity in our in vitro assays is not an appropriate parameter to explain clinical trial findings. Instead, investigating the impact of external factors, such as MPO, on each anticoagulant’s behavior may provide informative mechanistic insights into CAT treatment.
In this regard, it also has to be mentioned that peak and trough levels for rivaroxaban were based on once daily dosing of 20 mg. In the treatment of acute VTE, however, rivaroxaban is dosed with 15 mg twice daily for 3 weeks, and this initial phase of intensive anticoagulation may be particularly important in controlling a highly thrombogenic state such as CAT. In SELECT-D, superiority of rivaroxaban compared to dalteparin could not be explained by increased treatment persistence, because median treatment durations were 5.9 months (interquartile range, 2.5-6.0 months) and 5.8 months (3.0-6.0 months) in the rivaroxaban and dalteparin group, respectively, suggesting improved efficacy of rivaroxaban rather than improved efficiency. Although either not statistically significant or not defined as the primary endpoint, studies on edoxaban (HOKUSAI VTE Cancer) and apixaban (ADAM VTE and CARAVAGGIO) have also revealed lower VTE recurrence rates compared to dalteparin. We thus speculate that our findings obtained with rivaroxaban may also hold true for the other two oral factor Xa inhibitors. Until now, however, no specific safety and efficacy data from dedicated CAT trials are available for the direct oral thrombin inhibitor, dabigatran.

The discrepancies between MPO concentrations used in the in vitro experiments (Figure 2, Figure 3, and Figure 5) and those measured in the ex vivo inflammatory model (Figure 4) are a major concern of our study and require further critical discussion. MPO concentrations of ≥10 µg/mL were necessary to attenuate the anticoagulant activity of tinzaparin and dalteparin on 22Rv1-induced thrombin generation and fibrin clot formation (Figure 2 and Figure 3). Furthermore, the inhibitory effect of MPO was only partial despite LMWH trough levels. Although MPO plasma levels of up to 10 and 30 µg/mL have been reported in individual patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS), respectively, median MPO antigen levels in AML/MDS patients were in the range of 14 to 19 ng/mL as compared to 5 ng/mL in healthy controls. Thus, even in hematological malignancies, in which MPO is presumably released from transformed myeloid cells, average MPO plasma levels were multiple orders of magnitude lower than MPO concentrations applied in our in vitro experiments or measured in plasma supernatants from artificially stimulated whole blood.

In patients with solid tumors, normal granulocytes are most probably the predominant source of MPO, and increased serum MPO activity levels have been reported in patients with breast cancer. Because serum MPO activity levels were not associated with inflammatory tumor infiltrates, circulating granulocytes likely played a role. Paraneoplastic leukocytosis is a common finding in patients with cancer, and elevated leukocyte counts are predictive of VTE in hematology and oncology patients initiating ambulatory chemotherapy. More specifically, citrullinated histone H3, a biomarker of NET formation, predicts the risk of VTE and mortality in cancer patients, further implicating activated granulocytes in the pathogenesis of CAT and tumor progression. To the best of our knowledge, no data on MPO plasma levels are currently available for CAT patients with solid tumors and paraneoplastic leukocytosis. We aimed to generate inflammatory conditions in our tumor cell–induced thrombosis model by incubating whole blood with LPS or PMA, both of which have been shown to induce NET formation by granulocytes. Consistently, stimulation of whole blood with LPS or PMA significantly increased MPO plasma levels from <10 ng/mL to 200 to 400 ng/mL (Figure 4C), and the MPO concentration inversely correlated with the ability of tinzaparin to inhibit 22Rv1-induced thrombin generation in plasma supernatants (Figure 4D). Compared to experiments using exogenous MPO, much lower MPO concentrations were associated with an attenuated tinzaparin activity in the inflammation model. The reasons for this observation remain speculative, but may involve increased potency of the endogenously released, compared to the commercially supplied, MPO or the contribution of other factors generated during the stimulation process. In this regard, it is very well conceivable that activation of whole blood with unphysiologically high concentrations of LPS or PMA, such as those used in our study, results in a more non-specific type of cell death that is different from apoptosis, necrosis, or NETosis, with subsequent lysis of the cytoplasmic membrane and liberation of several other heparin-binding proteins not controlled for in our experiments. For instance, both monocytes and granulocytes contain a cationic antimicrobial protein also called heparin-binding protein (HBP) or azurocidin. Experiments using whole blood from MPO-deficient patients or MPO gene knock-out mice would be required to dissect the relative contributions of MPO and other cationic proteins to our observations. Still, even within the plasma supernatants from LPS-stimulated whole blood, we observed an intriguingly close inverse correlation between MPO antigen levels and the ability of tinzaparin to inhibit 22Rv1-induced thrombin generation (Figure S4). It is thus tempting to speculate that leukocyte-derived MPO is indeed capable of at least partially reversing the anticoagulant activity of LMWH in the context of cancer and systemic inflammation. Because plasma samples from PBS-, LPS-, or PMA-stimulated whole blood were subjected to double high-speed centrifugation prior to measuring 22Rv1-induced thrombin generation, we cannot comment on the effect of procoagulant (TF-bearing) microvesicles released during the stimulation process, which is another limitation of our study.

Interestingly, atypical expression of the normally myeloid-specific MPO gene by subsets of epithelial tumor cells has also been demonstrated in early to later stage ovarian cancer, suggesting that MPO production by (pre-)malignant tissues may not only drive cancer progression, e.g., through the induction of oxidation damage and oncogenic mutations, but also modulate the anticoagulant activity of LMWHs in the tumor microenvironment. We thus speculate that local MPO concentrations in tumors with intrinsic MPO expression and/or dense infiltration by PMNs may be significantly higher than systemic MPO plasma levels, which could account for at least some of the aforementioned discrepancies. Consistent with this hypothesis, the MPO concentration in phagosomes of neutrophils has been estimated at 1 to 2 mmol/L, corresponding to approximately 150 to 300 mg/mL and extracellular MPO antigen levels of 16 to 29 µg/mL have been measured in synovial fluids from patients...
with rheumatoid arthritis.\textsuperscript{58,59} Thus, excessive degranulation of neutrophils at sites of non-septic inflammation, as observed in malignant tissues and paraneoplastic blood clots, may result in local MPO concentrations that are similar to those applied in our in vitro experiments.

Partial inhibition of the anticoagulant activity of tinzaparin by MPO did not involve the catalytic properties of the enzyme, but required its structural integrity. This finding is consistent with electrostatic binding of the cationic MPO protein to the highly negatively charged glycosaminoglycan. In this regard, it is important to note that an earlier study has already demonstrated tight association of MPO derived from PMNs with standard and low molecular weight heparin that, in contrast to the association of heparin with the highly cationic protein cytochrome c, persisted at physiological conditions,\textsuperscript{60} thus providing a molecular basis for our functional observations. However, further studies are required to evaluate whether MPO also partially neutralizes the anticoagulant activity of shorter-chain LMWHs, such as enoxaparin, or the synthetic pentasaccharide fondaparinux. While rapid oxidation-mediated inactivation of MPO in the inflammatory microenvironment is not expected to affect its electrostatic properties,\textsuperscript{61,62} administration of heparin may actually further increase local and systemic MPO concentrations through the release of MPO from other negatively charged binding sites.\textsuperscript{60,63}

In summary, our proof-of-concept experimental study provides novel mechanistic insights into the pharmacology of anticoagulation in patients with CAT. Based on our findings, we hypothesize that (activated) PMNs are not only involved in the pathogenesis and prediction of cancer-associated VTE, but also capable of modulating the anticoagulant activity of LMWH. Paraneoplastic leukocytosis and diffuse infiltration by MPO-releasing granulocytes may thus render venous thrombi partially resistant to heparin therapy in the context of cancer and inflammation. However, additional (pre-)clinical studies involving other cell lines, animal models, and patient samples are required to prove this provocative hypothesis and to further resolve the discrepancies with regard to MPO concentrations used in vitro and measured in vivo, as extensively discussed before. Furthermore, considering that even at trough LMWH levels MPO only partially inhibited the anticoagulant activity of dalteparin and tinzaparin, additional mechanistic likely account for the superior efficacy of direct oral factor Xa inhibitors in CAT trials.

**ACKNOWLEDGMENTS**

The authors would like to thank Anna Klinke and Volker Rudolph for helpful discussions and valuable suggestions. Open access funding enabled and organized by Projekt DEAL.

**CONFLICTS OF INTEREST**

M. Voigtlaender has received travel support from Bristol-Myers Squibb and LEO Pharma. L. Beckmann, A. Schulenkorf, and B. Sievers declare no conflicts of interest relevant to the content of this article. C. Rolling has received travel support from Daiichi Sankyo and Pfizer. C. Bokemeyer has received personal fees for consultancy from Bayer, Bristol-Myers Squibb, and Sanofi. F. Langer has received personal fees for lectures or consultancy and/or research support from Aspen, Bayer, Boehringer-Ingelheim, Bristol-Myers Squibb, Daiichi Sankyo, LEO Pharma, Pfizer, and Sanofi.

**AUTHOR CONTRIBUTIONS**

M. Voigtlaender, L. Beckmann, A. Schulenkorf, and B. Sievers performed experiments and analyzed data. C. Rolling and C. Bokemeyer analyzed data and critically revised the manuscript. M. Voigtlaender and F. Langer designed the study, analyzed data, and wrote the first draft of the manuscript. All authors reviewed the final version of the manuscript and gave approval for its submission.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Voigtlaender M, Beckmann L, Schlenkorf A, et al. Effect of myeloperoxidase on the anticoagulant activity of low molecular weight heparin and rivaroxaban in an in vitro tumor model. J Thromb Haemost. 2020;18:3267-3279. https://doi.org/10.1111/jth.15075