Characterisation of antithrombin-dependent anticoagulants through clot waveform analysis to potentially distinguish them from antithrombin-independent inhibitors targeting activated coagulation factors

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ABSTRACT

Aims While antithrombin (AT)-independent inhibitors targeting thrombin or activated factor X have been assessed through clot waveform (CWA), there are no reports on assessment with respect to AT-dependent anticoagulants. The present study aims to characterise AT-dependent anticoagulants through CWA to distinguish them from AT-independent inhibitors.

Methods CWA was applied to the activated partial thromboplastin time (APTT) assay of plasma samples spiked with each of AT-dependent drugs (unfractionated heparin, enoxaparin and fondaparinux) and AT-independent drugs (rivaroxaban, apixaban, edoxaban, dabigatran, argatroban, hirudin and bivalirudin), which was performed using the CS-5100 or CN-6000 (Sysmex). The APTT-CWA data were automatically gained by the analyser program. The positive mode of clotting reaction curves was defined as the direction towards fibrin generation.

Results Regarding dose–response curves in AT-dependent anticoagulants, the maximum positive values of the first and secondary derivatives (Max1 and Max_p2, respectively) and the maximum negative values of the secondary derivative (Max_n2) seemed to drop to zero without making an asymptotic line, consistent with the irreversibility. Such a feature was observed also in hirudin, as reported previously. Notably, the symmetric property of Max1 peaks in the waveforms was distorted dose dependently in AT independent but not AT-dependent drugs. A plot of Max_p2 logarithm versus Max_n2 logarithm was linear. The slope was about 1 in AT-dependent drugs while that was more than 1 in AT-independent drugs. These features made it possible to distinguish AT-dependent and AT-independent drugs.

Conclusions The results aid in further understanding of the pharmacological aspects of anticoagulation and in screening of candidates for novel anticoagulants.

INTRODUCTION

Clot waveform analysis (CWA) has been reported to extend the interpretation of measurement curves for coagulation time to provide additional information about coagulation abnormalities and disorders.[1–6] The parameters are obtained from successive derivatives of the clotting reaction curve, mathematically dissecting the coagulation cascade into each reaction for activation of coagulation factors. If the coagulation analyser has a program for automatic analysis, CWA is available in clinical laboratories routinely handling coagulation-related tests such as activated partial thromboplastin time (APTT) and prothrombin time (PT). CWA is based on clotting reaction triggered by APTT or PT reagents that are less close to physiological triggers than reagents used in thromboelastography (TEG), rotational thromboelastometry (ROTEM) and thrombin generation assay (TGA), which are used for evaluation of global haemostatic potential.[7 8] On the other hand, as CWA requires no specialised instruments in contrast to TEG, ROTEM and TGA, it is convenient for practical spread in clinical laboratories.[1–3]

We have reported the usability of CWA for assessment of in vitro effects of direct thrombin inhibitors (DTIs), dabigatran and argatroban, as well as direct activated factor X (FXa) inhibitors, rivaroxaban, apixaban and edoxaban.[9] In addition, distinct features of bivalent DTIs, hirudin and bivalirudin have been demonstrated through CWA.[10] These drugs directly inhibit thrombin or FXa independently of antithrombin (AT).

While AT-independent inhibitors targeting serine protease activity of activated coagulation factors have been assessed through CWA, there are no reports on assessment with respect to AT-dependent anticoagulants, unfractionated heparin (UFH), low-molecular-weight heparin (LMWH) such as enoxaparin and fondaparinux. The distinct features of a reversible bivalent DTI, bivalirudin and an irreversible bivalent DTI, hirudin, which have been revealed through CWA, result putatively from a difference in the reversibility.[10] The AT-dependent anticoagulants are thought to cause irreversible inhibition because they act via a covalent mechanism involving acylation of active sites of targeted serine protease such as thrombin and FXa.[11] However, more recently, reversible covalent DTIs have been reported, which exert transient acylation.[12] It is of considerable interest whether laboratory findings can reveal properties related to the irreversibility of AT-dependent anticoagulants. In addition, it is of

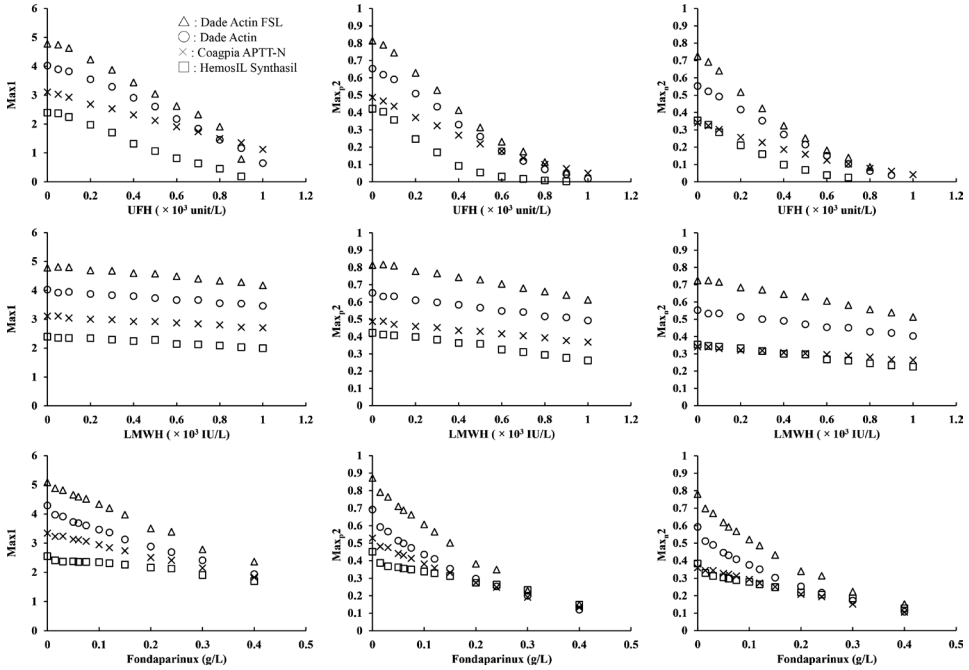


Figure 1 Antithrombin-dependent anticoagulants dose dependently decrease the maximum positive values of the first derivative (Max1) as well as the maximum positive and negative values of the second derivative (Max$_p$2 and Max$_n$2). Max1, Max$_p$2 and Max$_n$2 correspond to the maximum coagulation velocity, acceleration and deceleration, respectively. For each activated partial thromboplastin time (APTT) reagent, Max1, Max$_p$2 and Max$_n$2 were decreased in plasma spiked with unfractionated heparin (UFH), low-molecular-weight heparin (LMWH) or fondaparinux dose dependently.

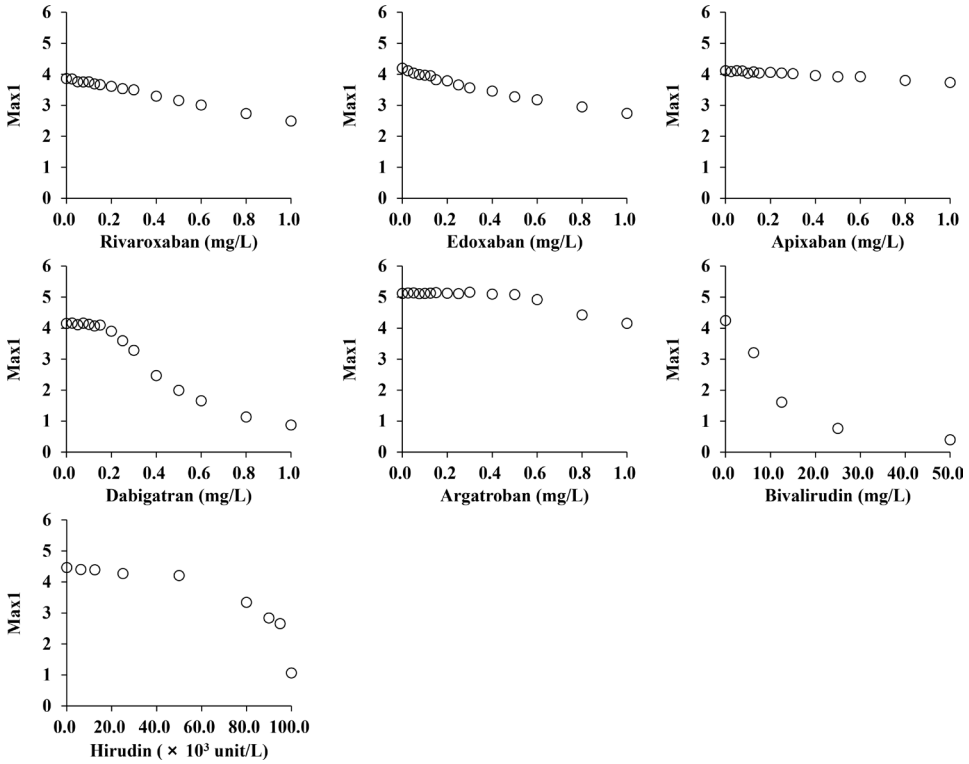


Figure 2 Antithrombin-independent reversible inhibitors targeting activated factor X (rivaroxaban, apixaban and edoxaban) or thrombin (dabigatran, argatroban and bivalirudin) and an antithrombin-independent irreversible thrombin inhibitor (hirudin) dose dependently decrease the maximum positive values of the first derivative (Max1), which correspond to the maximum coagulation velocity. The results obtained using the activated partial thromboplastin time (APTT) reagent, Dade Actin FSL, are shown. Experiments using other APTT reagents provided similar observations (data not shown). The maximum positive and negative values of the second derivative (Max$_p$2 and Max$_n$2), which correspond to the maximum coagulation acceleration and deceleration, respectively, were also decreased in plasma spiked with each drug dose dependently, similarly to Max1 (data not shown).

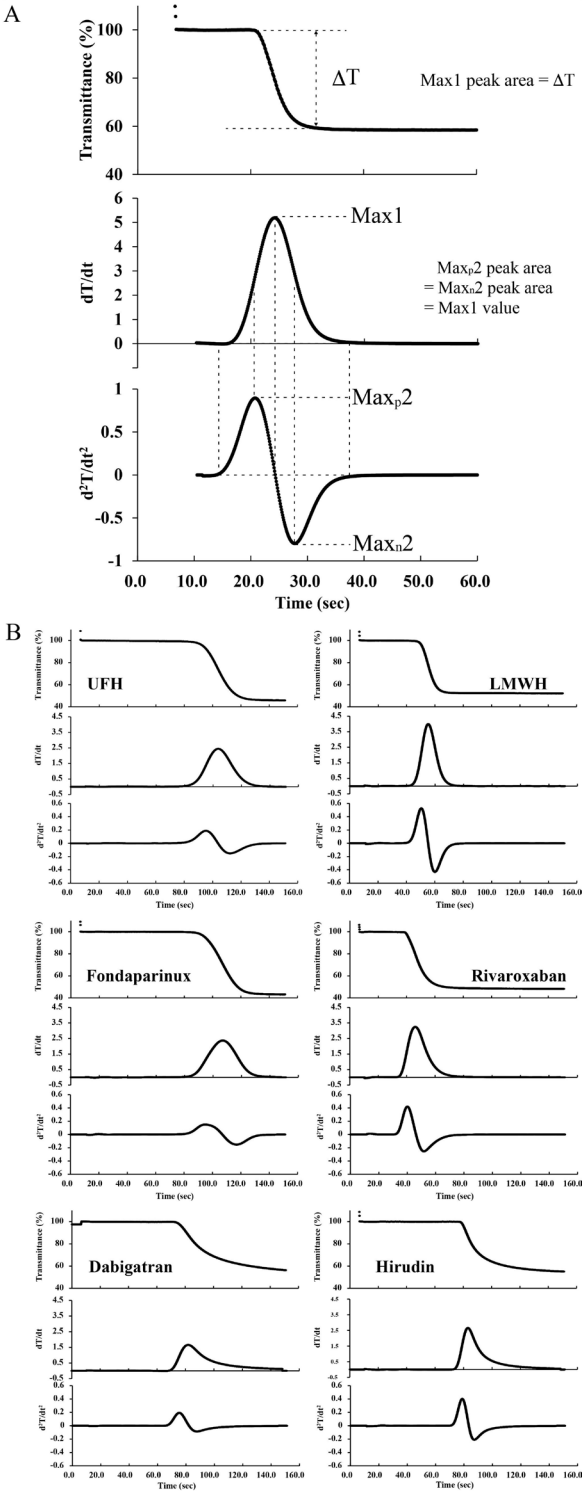


Figure 3 The clot waveforms and parameters are obtained from successive derivatives of the clotting reaction curves. (A) The representative results using normal plasma are shown. (B) The representative results using plasma spiked with each of three antithrombin-dependent anticoagulants, unfractionated heparin (UFH), low-molecular-weight heparin (LMWH) and fondaparinux and three antithrombin-independent anticoagulants, rivaroxaban, dabigatran and hirudin, are shown. ΔT, the change in a transmittance; Max1, the maximum positive values of the first derivative; Max_p2, the maximum positive values of the second derivative; Max_n2, the maximum negative values of the second derivative.

critical importance whether AT-dependent anticoagulants are distinguishable from irreversible AT-independent drugs such as hirudin.

The present study aimed to characterise AT-dependent anticoagulants through CWA to distinguish them from AT-independent inhibitors targeting activated coagulation factors from the perspective of laboratory haematology. As a result, the CWA parameter profiles demonstrated differences not only in the reversibility but also in the AT dependence, potentially distinguishing the AT-dependent and AT-independent drugs. These obtained findings are expected to aid in further understanding of the pharmacological aspects of anticoagulation and in screening of candidates for novel anticoagulants.

METHODS

Sample preparation

The heparin sodium solution at 1×10^7 unit/L (AY Pharmaceuticals, Tokyo, Japan), which is derived from porcine intestinal mucosa, was used as UFH. The enoxaparin sodium solution at 1×10^8 IU/L (Sanofi, Paris, France) was used as LMWH. Fondaparinux (Merck KGaA, Darmstadt, Germany) was solved in H_2O at 20 g/L. The stock solutions of fondaparinux were aliquoted and stored at −80°C. UFH, LMWH and fondaparinux were diluted in series and applied to spiking CRYOcheck Pooled Normal Plasma (Precision BioLogic, Dartmouth, Canada) at the final concentrations ranging from 0 unit/L to 1.0×10^3 unit/L, from 0 IU/L to 7×10^3 IU/L, and 0 g/L to 0.4 g/L, respectively.

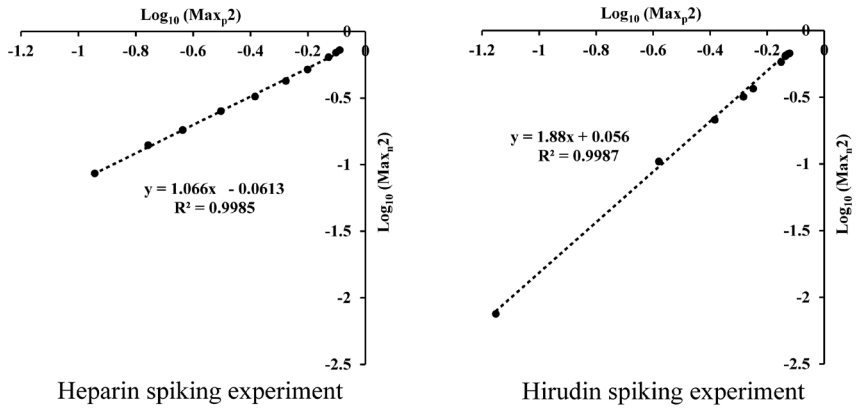
The measurement of the AT activity level in the plasma used in the present study was performed with the commercially available kit, Chromorate AT III (C), based on the chromogenic method using a synthetic substrate of thrombin, H-D-Phe-Ala (NCH_3)-Arg-pNA (LSI Medience, Tokyo, Japan) in the fully automated clinical laboratory analyser, STACIA (LSI Medience). The measurement of the AT antigen level in the plasma used in the present study was performed with the commercially available kit, LPIA ATIII, based on the latex aggregation method using polyclonal antibodies specific to AT (LSI Medience) in STACIA. The activity and antigen levels were 89.4% and 30.5 mg/dL, respectively, within normal limits (83%–118% and 24–36 mg/dL, respectively).

Preparation of plasma samples spiked with each of AT-independent drugs (rivaroxaban, apixaban, edoxaban, dabigatran, argatroban, hirudin and bivalirudin) was done as reported previously.[9 10]

Activated partial thromboplastin time-clot waveform analysis

APTT measurements and APTT-CWA were performed in the automated coagulation system, CS-5100 or CN-6000 (Sysmex, Kobe, Japan). Dade Actin (Siemens Healthineers, Erlangen, Germany), Dade Actin FSL (Siemens Healthineers), Coagpia APTT-N (Sekisui Medical, Tokyo, Japan) and HemosIL SynthASil (Instrumentation Laboratory, Bedford, Massachusetts, USA) were used in APTT measurement assays. For CWA, the first and second derivatives were automatically gained using the CS-5100 or CN-6000 programme. In the present study, the positive mode of clotting reaction curves was defined as the direction towards fibrin generation. Therefore, the maximum positive value of the first derivative (Max1) corresponds to the maximum coagulation velocity while the maximum positive and negative values of the secondary derivative (Max_p2 and Max_n2, respectively) correspond to the maximum coagulation acceleration and deceleration, respectively.

A



B

	target	reversibility	slope in the plot of Max_p2 logarithm versus Max_n2 logarithm							
			reagent A	reagent B	reagent C	reagent D	mean	SD	mean*	SD*
AT-dependent drugs										
heparin (UFH)	thrombin, FXa, etc.	irreversible	1.066	0.984	0.918	0.808	0.944	0.094	0.989	0.0605
enoxaparin (LMWH)	FXa, etc.	irreversible	1.219	1.114	0.911	0.971	1.054	0.121	1.081	0.128
fondaparinux	FXa	irreversible	0.994	1.044	0.8395	1.115	0.998	0.101	0.959	0.087
AT-independent drugs										
rivaroxaban	FXa	reversible	1.662	1.685	1.887	2.076	1.828	0.168	1.745	0.101
apixaban	FXa	reversible	1.538	1.359	1.353	1.564	1.454	0.098	1.417	0.0858
edoxaban	FXa	reversible	1.633	1.489	1.771	2.558	1.863	0.414	1.631	0.115
dabigatran	thrombin	reversible	1.61	1.938	2.072	1.886	1.877	0.168	1.873	0.194
argatroban	thrombin	reversible	1.696	1.412	1.886	2.312	1.827	0.327	1.665	0.195
hirudin	thrombin	irreversible	1.88	1.76	2.028	1.579	1.812	0.165	1.889	0.110
bivalirudin	thrombin	reversible	1.923	1.715	1.577	2.194	1.852	0.233	1.738	0.142

Figure 4 Distinction between antithrombin (AT)-dependent and AT-independent drugs is exhibited by a functional relation between the maximum coagulation acceleration and deceleration. (A) A plot of the maximum positive value of the second derivative (Max_p2) logarithm versus the maximum negative value of the second derivative (Max_n2) logarithm in each of AT-dependent and AT-independent drugs was linear. Max_p2 and Max_n2 correspond to the maximum coagulation acceleration and deceleration, respectively. The plots based on heparin spiking and hirudin spiking experiments with the activated partial thromboplastin time (APTT) reagent, Dade Actin FSL, are shown as examples of AT-dependent and AT-independent drugs, respectively. (B) The slope in the plot of Max_p2 logarithm versus Max_n2 logarithm in each drug is shown. Mean* and SD* indicate the mean and SD of the slope values in the cases of the APTT reagents A, B and C, respectively. The reagents A, B, C and D indicate Dade Actin FSL, Dade Actin, Coagpia APTT-N and HemosIL SynthASil. FXa, activated factor X; LMWH, low-molecular-weight heparin; UFH, unfractionated heparin.

Statistical analyses

Linear regression was performed using the Microsoft Excel software packaged in the Microsoft Office (Microsoft).

RESULTS

The irreversibility of AT-dependent anticoagulants revealed by the dose–response curves to which the Hill plot analysis is inapplicable

In UFH, LMWH and fondaparinux, dose-dependent decreases in Max1, Max_p2 and Max_n2 were almost linear but not downward convex or reverse sigmoid, seeming to drop to zero without making an asymptotic line (figure 1). The Hill plot analysis was inapplicable to theses curves. Such a feature was observed in an irreversible AT-independent thrombin inhibitor, hirudin, as reported previously (figure 2).[10] Other drugs, which are reversible AT-independent inhibitors targeting FXa or thrombin, exhibited the dose–response curves to which the Hill plot analysis is applicable, as reported previously (figure 2).[9 10] Thus, the irreversibility of AT-dependent anticoagulants was characterised by the dose–response curves regarding Max1, Max_p2 and Max_n2 to which the Hill plot analysis is inapplicable.

Distinction between AT-dependent and AT-independent drugs exhibited by a functional relation between the maximum coagulation acceleration and deceleration

As the irreversibility characterised by the dose–response curves to which the Hill plot analysis is inapplicable was observed not only in the three AT-dependent anticoagulants but also in hirudin, it did not allow us to distinguish them. It was of critical importance whether CWA parameters can provide findings to distinguish AT-dependent and AT-independent drugs. Through our careful observations, it was found out that the symmetric property of Max1 peaks in the waveforms was distorted dose dependently in AT-independent but not AT-dependent drugs (figure 3).

This difference seemed to be related to profiles of Max_p2 and Max_n2. A plot of Max_p2 logarithm versus Max_n2 logarithm in each drug was linear (figure 4A). Roughly, the slope was about 1 in AT-dependent drugs while that was more than 1 in AT-independent drugs (figure 4B). The slope was raging from 0.84 to 1.22 in AT-dependent drugs. The mean values were 0.944 in UFH, 1.05 in LMWH and 0.998 in fondaparinux. On the other hand, the slope was raging from 1.35 to 2.56 in AT-independent drugs. The mean values were 1.83 in rivaroxaban, 1.45 in apixaban, 1.86 in edoxaban, 1.88 in dabigatran, 1.83 in argatroban,1.81 in hirudin and 1.85 in

bivalirudin. With respect to the activator of the intrinsic pathway of coagulation, the HemosIL SynthASil contains silica while the other reagents contain ellagic acid. Despite the difference, the mean values of the slope among the reagents other than the HemosIL SynthASil were similar to those among all the reagents (figure 4B).

The slope equaling 1 means linear relation between Max_p2 and Max_n2, keeping the symmetric property of Max1 peak shapes even with increasing drug concentrations. On the other hand, the slope not equaling 1 means non-linear relation between Max_p2 and Max_n2, distorting the symmetric property of Max1 peak shapes dependently on drug concentrations. These features made it possible to distinguish AT-dependent and AT-independent drugs. In the case of Dade Actin DSL, the mean±SD of the slope values among AT-dependent drugs was 1.093±0.094 while that was 1.706±0.132 among AT-independent drugs. In the case of Dade Actin, the mean±SD among AT-dependent drugs was 1.047±0.053 while that was 1.623±0.194 among AT-independent drugs. In the case of Coagpia APTT-N, the mean±SD among AT-dependent drugs was 0.89±0.035 while that was 1.796±0.236 among AT-independent drugs. In the case of HemosIL SynthASil, the mean±SD among AT-dependent drugs was 0.965±0.125 while that was 2.024±0.344 among AT-independent drugs. Based on these, the cut-off values of the slope to distinguish AT-dependent and AT-independent drugs were determined to be 1.38, 1.26, 1.24 and 1.39 in Dade Actin DSL, Dade Actin, Coagpia APTT-N and HemosIL SynthASil, respectively.

DISCUSSION

TEG, ROTEM and TGA are methods to evaluate global haemostatic potential, reflecting the comprehensive processes of coagulation.[7 8] Although studies concerning in vitro effects of AT-dependent and AT-independent anticoagulants have been made using TEG, ROTEM and TGA, there are no reports focusing on whether the drug is reversible or irreversible or on whether the drug does depend on AT or not.[13–25]

To the best of our knowledge, this is the first report to demonstrate that CWA parameter profiles make it possible to assess the AT dependence as well as the reversibility of anticoagulants. Irreversible binding of inhibitors inactivates and consumes targeted enzymes. Complete depletion of enzyme molecules gives rise to failure in the reaction. This may explain that Max1, Max_p2 and Max_n2 seemed to drop to zero without making an asymptotic line in AT-dependent anticoagulants and hirudin. Hirudin is a non-covalent but irreversible DTI because of its bivalence leading to tight binding to thrombin without cleavage by thrombin.[26] On the other hand, as AT-dependent anticoagulants act via a covalent mechanism involving acylation of active sites of targeted serine protease, it is plausible that AT-dependent anticoagulants exert irreversible effects.[11] However, there are also reversible covalent DTIs which exert transient acylation, as reported more recently.[12] It is of considerable interest how to assess the reversibility or irreversibility of anticoagulants regardless of whether they act via covalent or non-covalent mechanisms. The present study demonstrated the usefulness of dose–response curves regarding CWA parameters to ascertain whether anticoagulants are reversible or irreversible.

Since both the Max_p2 peak area and the Max_n2 peak area equal the Max1 peak value in theory, dose-dependent discrepancy in reduction between Max_p2 and Max_n2 induced by the drug results in a difference in the time length between the period from the start point to the top and the period from the top to the end point in the Max1 peak. Thus, a functional relation between Max_p2 corresponding to the maximum coagulation acceleration and Max_n2 corresponding to the maximum coagulation deceleration influences the symmetric property of the peak shape of Max1 corresponding to the maximum coagulation velocity. The slope being 1 in a plot of Max_p2 logarithm versus Max_n2 logarithm, which was observed in AT-dependent drugs, means that Max_n2 is directly proportional to Max_p2. In this case, Max_n2 is reduced similarly to Max_p2, leading to similar prolongation of the time length in the period from the start point to the top and the period from the top to the end point in the Max1 peak. On the other hand, the slope being more than 1, which was observed in AT-independent drugs, means that Max_n2 reduction is discrepant from Max_p2 reduction, leading to different prolongation of the time length in the period from the start point to the top and the period from the top to the end point in the Max1 peak. This results in distorting the symmetric property of the Max1 peak. Taken together, the functional relation between Max_p2 and Max_n2 made it possible to distinguish AT-dependent and AT-independent drugs. Although there are differences among APTT reagents used for CWA assays, the cut-off values of the slope in a plot of Max_p2 logarithm versus Max_n2 logarithm for distinguishing them range from 1.24 to 1.39 according to the present study.

The mechanisms by which such a difference in the functional relation between Max_p2 and Max_n2 between AT-dependent and AT-independent drugs occurs remain to be elucidated. AT-dependent anticoagulants target only the free forms of activated coagulation factors while AT-independent inhibitors target both the free forms and the complex forms such as the prothrombinase complex consisting of FXa, FVa and phospholipids. This distinction seemingly gives rise to different impacts on the cascade and feedback of the coagulation pathway and might be related to the difference in the functional relation between Max_p2 and Max_n2. Waveforms of Max1 are influenced by coagulation acceleration, which corresponds to the rate of thrombin generation by FXa taking the free form or the prothrombinase complex form. FX activation by the Xase complex consisting of FVIIIa and FIXa is thought to reflect thrombin positive feedback more strongly than that by the Xase consisting of tissue factor and FVIIa. Since APTT reagent-triggered clotting involves thrombin generation via FX activation by FVIIIa and FIXa, the waveforms of Max1 as well as Max_p2 and Max_n2 are affected, likely reflecting alteration in thrombin positive feedback due to anticoagulation. Lines of basic evidence and findings from clinical samples should be accumulated to clarify whether and how inhibition of both the free and complex forms of activated coagulation factors can give rise to the discrepancy in reduction between the maximum coagulation acceleration and deceleration corresponding to Max_p2 and Max_n2, respectively.

With respect to the activator of the intrinsic pathway of coagulation, the HemosIL SynthASil and the other APTT reagents contain silica and ellagic acid, respectively. It has been reported that impacts of silica on the cascade and feedback of the coagulation pathway are different from those of ellagic acid.[27–32] Although such differences among APTT reagents should be considered for application of CWA to assessment of anticoagulation, no differences were observed between the HemosIL SynthASil and the other reagents in the present study.

To make CWA assays closer to physiological conditions, it might be better to use a small amount of thrombin (or FXa and prothrombin) or a small amount of tissue factor instead of APTT reagents.[33] Such modifications would be undertaken as a next step for further understanding of the properties of anticoagulation.

In conclusion, the present study proved that CWA parameter profiles are useful in assessment of the AT dependence as well as

the reversibility of anticoagulants. The results aid in further understanding of the pharmacological aspects of anticoagulation and in screening of candidates for novel anticoagulants.

Take home messages

- Regarding dose–response curves in antithrombin (AT)-dependent anticoagulants, the clot waveform analysis parameters seemed to drop to zero without making an asymptotic line, consistent with the irreversibility.
- The symmetric property of peaks obtained by the first derivative of clotting reaction curves was distorted dose dependently in AT-independent but not AT-dependent drugs.
- The functional relation between the maximum positive and negative values of the secondary derivative could distinguish AT-dependent and AT-independent drugs.
- The results aid in further understanding of the pharmacological aspects of anticoagulation and in screening of candidates for novel anticoagulants.

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Contributors MW and YF designed the study, analysed and interpreted the data, and wrote the manuscript. MW conceived the study. YF and SN performed the experiments. All authors discussed the data and critically reviewed and revised the manuscript. All authors have given final approval for this version of the manuscript to be published.

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