Studies on the Different Modes of Action of the Anticoagulant Protease Inhibitors DX-9065a and Argatroban

I. EFFECTS ON THROMBIN GENERATION*‡

The present study began with mathematical modeling of how inhibitors of both factor Xa (fXa) and thrombin affect extrinsic pathway-triggered blood coagulation. Numerical simulation demonstrated a stronger inhibition of thrombin generation by a thrombin inhibitor than a fXa inhibitor, but both prolonged clot time to a similar extent when they were given an equal dissociation constant (30 nM) for interaction with their respective target enzymes. These differences were then tested by comparison with the real inhibitors DX-9065a and argatroban, specific competitive inhibitors of fXa and thrombin, respectively, with similar Ki values. Comparisons were made in extrinsically triggered human citrated plasma, for which endogenous thrombin potential and clot formation were simultaneously measured with a Wallac multilabel counter equipped with both fluorometric and photometric detectors and a fluorogenic reporter substrate. The results demonstrated stronger inhibition of endogenous thrombin potential by argatroban than by DX-9065a, especially when coagulation was initiated at higher tissue factor concentrations, while argatroban appeared to be slightly less potent in its ability to prolong clot time. This study demonstrates differential inhibition of thrombin generation by fXa and thrombin inhibitors and has implications for the pharmacological regulation of blood coagulation by the anticoagulant protease inhibitors.

The purpose of the current study is to investigate the differences in the modes of action of two anticoagulant protease inhibitors, DX-9065a (1) and argatroban (2). DX-9065a is a synthetic fXa inhibitor currently under clinical development. Argatroban is a synthetic thrombin inhibitor, which is already approved in Japan and the United States as an anticoagulant agent. Despite targeting different enzymes, the two inhibitors are similar in that they are synthetic, low molecular weight compounds, are monospecific to one of the coagulation cascade proteases, and can inhibit their target enzymes directly and competitively. Moreover, they have similar Ki values for inhibition of the respective target enzymes: Ki values are 41 nM for fXa with DX-9065a (1) and 39 nM for thrombin with argatroban (2).

The coagulation cascade comprises multiple sequential steps of protease reactions, and it is not intuitively obvious how the kinetics of the activation of each step contributes to the overall cascade. In the current study, as an attempt to better understand the differences between fXa inhibitors and thrombin inhibitors, a mathematical model of blood coagulation has been developed to simulate actions of these compounds. Computer modeling shows that a thrombin inhibitor gives a stronger inhibitory effect on thrombin generation than an fXa inhibitor. However, the two types of inhibitor prolong clot time to a similar extent when coagulation is triggered with an extrinsic pathway stimulus of the same intensity.

In the experimental portions of the current study, a microplate method was developed to examine thrombin generation and clot formation simultaneously during the course of plasma coagulation. The method utilized a fluorogenic reporter substrate, Z-GGR-AMC (3, 4), in combination with a Wallac multilabel counter equipped with both fluorometric and photometric detectors. This methodology was used to compare the abilities of DX-9065a and argatroban to inhibit thrombin generation as well as to prolong clot time during extrinsically initiated plasma coagulation. It was shown that, while the two compounds prolonged clot time to a comparable extent, argatroban exerted a much stronger inhibitory effect on thrombin generation than did DX-9065a. The results were in good agreement with those predicted by the numerical simulation described herein.

EXPERIMENTAL PROCEDURES

The Mathematical Model—The mathematical model used in the current study is presented as the corresponding reaction scheme (Fig. 1). The differential equation derived from the reaction scheme and rate constants used for numerical simulation of the model are given in Tables S1 and S2, which are supplied as Supplementary Data and can be seen in the online version of this article at http://www.jbc.org. All of the kinetic species were evaluated by the simulation procedures; among a total of 32 species evaluated 24 are given as separate ordinary differential equations, and the remaining eight can be calculated from equations for the conservation of the initial concentration of components added to the reaction mixture. The model was solved numerically by integrating the differential equations using a computer program, PDESOL v2.0, from Numerica (Dallas, TX), which was executed on a Windows-based personal computer.

Materials—DX-9065a was obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Argatroban was obtained from Mitsubishi Pharma Corporation (Tokyo, Japan). Human thrombin was purchased from Calbiochem (San Diego, CA). Bovine albumin and PPACK (D-Phe-Pro-Arg-chloromethylketone) were from Sigma. Human recombinant thromboplastin (Recombiplastin) was from Instrumentation Laboratory (Barcelona, Spain). Z-GGR-AMC and AMC were from Bachem (Bubendorf, Switzerland).

Preparation and Storage of Human Citrated Plasma—Blood was...
withdrawn from healthy volunteers on an anticoagulant, 0.13 M trisodium citrate (9 parts of blood to 1 part of citrate). Platelet-poor plasma, obtained as the supernatant after repeating two centrifugations at 6000 g for 15 min at 4 °C, was pooled, distributed into plastic tubes, and stored at -80 °C until use.

**Preparation of the Stock Thromboplastin Solution**

A commercially available thromboplastin reagent, Recombiplastin, was used to trigger plasma coagulation. The reagent was obtained in lyophilized form from the manufacturer and was reconstituted in 0.5 M CaCl₂. After incubation at 37 °C for 30 min, the reconstituted thromboplastin solution was distributed into small plastic tubes and stored at -80 °C. Frozen samples were stored for 2 months without any discernable deterioration. Before use, the frozen stock solutions were thawed by incubation at 37 °C for 10 min and further diluted with 0.5 M CaCl₂ at a ratio of 1/3, 1/10, 1/30, 1/100, or 1/300. Preparation of thromboplastin solutions by these procedures maintained the final CaCl₂ concentration at 25 mM following a 20-fold dilution into the assay mixture.

**A Microplate Method to Monitor Thrombin Generation and Clot Formation**

Coagulation was performed in a microtiter plate (96-well, half-area plate, Corning) in a final mixture of 100 μl, which was prepared by the following procedures. To 6.5-μl amounts (0.05 volume of the total) of the diluted solution of test compounds was added 117 μl (0.9 volume) of human citrated plasma supplemented with a fluorogenic thrombin substrate, Z-GGR-AMC, at a final concentration of 250 μM. After preincubating the resulting mixtures for 3 min at 37 °C, 6.5 μl was taken from each of the 130-μl mixtures and gently transferred to other vacant wells on the same microtiter plate. This additional transfer effectively removed any bubbles that might have formed during preparation of the mixtures, which could, if present, cause erroneous signals in the following measurements.

The microtiter plate was read with a microplate reader equipped with both fluorometric and photometric detectors (Wallac 1420 ARVO sx Multilabel Counter, Amersham Biosciences) thermoregulated at 37 °C. The fluorometric measurements (ex 390 nm, em 460 nm) of thrombin-liberated AMC from the substrate were made to monitor changes in thrombin activity during the course of coagulation. In addition, photometric measurements (390 nm) were also performed to assess clot formation during the course of coagulation. The fluorometric and photometric measurements were alternated with a 40-s cycle for 1 h.

Twelve-channel pipettes were used as liquid handling devices in these microplate assays. As the pipette can handle 12 wells at once, one can manipulate a maximum of 12 wells in a single assay run. Typically, in each assay run, single comparisons of DX-9065a and argatroban were made at six escalating concentrations: 0, 0.375, 0.75, 1.5, 3, and 6 nM.

**Determination of Km and kcat for Hydrolysis of Z-GGR-AMC by Active Site-titrated Thrombin**

The active site titrated-thrombin, which had been prepared according to the previous literature (4), was used to determine Km and kcat values of 172 μM and 1.2 s⁻¹, respectively.

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**Table I**

<table>
<thead>
<tr>
<th>Coagulation factors</th>
<th>Initial concentrations a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>7900 nM</td>
</tr>
<tr>
<td>Factor II</td>
<td>1400 nM</td>
</tr>
<tr>
<td>Factor V</td>
<td>20 nM</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>0.7 nM</td>
</tr>
<tr>
<td>Factor IX</td>
<td>90 nM</td>
</tr>
<tr>
<td>Factor X</td>
<td>170 nM</td>
</tr>
</tbody>
</table>

a The values are derived from Ref. 9, except for fibrinogen (10).

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FIG. 1. Enzyme reactions used in the model. Colons indicate complex formation, and the suffixes a and i denote activated and irreversibly inactivated species, respectively.
Calculation of Endogenous Thrombin Potential (ETP)—The fluorometric and photometric data from single wells were retrieved and analyzed by the Excel spreadsheet program (Microsoft). Thrombin generation curves were obtained from each of the wells by the following calculation. 1) For correction of color quenching in plasma samples, the fluorescence signals were calibrated against a known concentration of free AMC (20 nM) in the same plasma preparations as used in the thrombin generation assays. Among the plasma preparations examined, the lot-to-lot variations of the coefficient of molar fluorescence intensity ranged from 3.5 to 3.9 counts/nM AMC. 2) Thrombin amidolytic activities were calculated from the equation, 
\[
E_T = \frac{v}{K_m} + \frac{S_0}{k_{cat}} \times \frac{S_0}{v}
\]
where \(v\) denotes the amidolytic velocity calculated as the first derivative of the AMC-calibrated fluorescent signal, \(S_0\) is the initial concentration of the thrombin substrate Z-GGR-AMC (250 μM), and \(K_m\) and \(k_{cat}\) are the Michaelis-Menten constant and the catalytic rate constant for the hydrolysis of the substrate, respectively. 3) Correction for residual activity of the 2-macroglobulin-thrombin complex was made on an Excel spreadsheet by a Solver-assisted numerical calculation procedure according to the algorithm described by Hemker and Beguin (6). 4) ETP was determined as the time-integral of the thrombin generation curves for each of the fluorometric recordings and averaged over triplicate determinations. 5) The IC_{50} values for inhibition of ETP by DX-9065a or argatroban were determined from linear interpolation between the dose-escalation data points.

Determinations of Clot Time—Clot time is defined as the time at which solution turbidity exceeds half-maximal values, i.e. \((\text{maximum turbidity} + \text{minimum turbidity})/2\), due to clot formation. In accordance with this definition, clot time was determined by a linear interpolation method between the data points in the ascending phase of each photometric recording. Further, the anticoagulant concentration required to double the clot time, CT_{2}, was determined from each set of the dose-escalation data, which were obtained from a single assay run, and usually consisted of 12 assay wells. A linear interpolation method was used to determine CT_{2} values from the data, and the results were averaged over triplicate runs. All of these calculations were performed semiautomatically by the Excel spreadsheet program (Microsoft).

RESULTS

The Mathematical Model—The enzyme reactions used in the current model are similar to those described earlier for the
mathematical modeling of extrinsically triggered blood coagulation (7, 8), except that antithrombin-III and fibrinogen/fibrin are additionally incorporated into the model, but the involvement of meizothrombin described in the previous literatures (7, 8) is not considered in the current study. The reaction scheme used in the current model is summarized in Fig. 1. The model consists of a set of coagulation factors including factors II, V, VII, VIII, IX, and X, TF, fibrinogen/fibrin, and antithrombin-III. In addition to these coagulation factors, an inhibitor that can exert simple, specific, and reversible inhibition of either FXa or thrombin is considered, where an equal dissociation constant of 30 nM is assumed for its binding to active sites of either enzyme.

The initial concentrations of the procoagulant components are given in Table I, and the kinetic constants for each enzyme reaction are given in Table S2 (supplied as Supplementary Data in the on-line version of this article at http://www.jbc.org). As indicated in these tables, the parameter values used in the current study are largely derived from the previous literature (8), which also describes a mathematical model of an extrinsic pathway-triggered blood coagulation. The numerical calculation was started by the addition of a small amount of TF:VIIa complex (5 pM), which is assumed to be fully formed at the start of coagulation. The model was formulated to a system of algebraic and differential equations and was solved numerically as described under “Experimental Procedures.”

Fig. 2 shows the results of numerical simulation of the model. The FXa inhibitor and the thrombin inhibitor both prolonged the initiation phase of the thrombin activity curve to a similar extent (Fig. 2, A and B). Correspondingly, the two inhibitors similarly prolonged clot time as shown in Fig. 2, C and D. These results indicate that the inhibitors have comparable anticoagulant potencies under the given conditions. They differed significantly, however, in the ability to reduce thrombin generation; the FXa inhibitor did not suppress thrombin activity peaks as effectively as did the thrombin inhibitor (refer to Fig. 2, A and B).

Effects on Thrombin Generation and Clot Formation—To investigate the possible differential effects of the FXa and thrombin inhibitors predicted by the mathematical model described herein, a microplate method was developed to examine thrombin generation and clot formation simultaneously during human plasma coagulation. A Wallac multilabel counter equipped with both fluorometric and photometric detectors was used for these measurements. For the thrombin activity assay, a fluorogenic substrate, Z-GGR-AMC, was monitored fluorometrically (ex 390 nm, em 460 nm) to follow hydrolysis of the substrate continuously during the course of coagulation, whereas photometric detection (390 nm) was performed simultaneously to determine clot formation independently of the fluorometric measurements. The fluorometric data were then mathematically reduced to calculate ETP according to the algorithm described by Hemker et al. (5, 6) (see “Experimental Procedures”). Fig. 3 compares the effects of DX-9065a and argatroban on thrombin generation as well as on clot formation following extrinsic stimulation of human citrated plasma. In the absence of inhibitors, the thrombin generation curve displayed an initiation phase followed by an explosive propagation phase, as was repeatedly shown in the previous studies (3–6). DX-9065a and argatroban showed a comparable increase in coagulation time (Fig. 3, C and D), suggesting that they exhibited similar anticoagulant potencies under the conditions employed; however, the attenuation of thrombin peaks by DX-9065a was less evident than that by argatroban (Fig. 3, A and B), in good agreement with the numerical simulation results given in Fig. 2, A and B.

FIG. 4. Relationship between ETP and CT during the extrinsically triggered plasma coagulation in the presence of DX-9065a or argatroban. Coagulation was triggered by the addition of 0.05 volume of thromboplastin solution diluted at 1/3 (A), 1/10 (B), 1/30 (C), 1/100 (D), or 1/300 (E), as described under “Experimental Procedures.” The final solutions contained DX-9065a (●) or argatroban (○) at the indicated concentrations (μM). Average values for ETP and clot time were obtained from the time-course data for thrombin generation and clot formation, respectively. Values represent means ± S.D. of triplicate determinations.

Fig. 4 represents the correlation between the mean values of ETP and clot time for triplicate measurements. From these data, both the IC50 values for inhibition of ETP, ETP IC50, and
TABLE II
Differential effects of DX-9065a and argatroban on thrombin generation and clot formation

ETP IC₅₀ represents IC₅₀ values for inhibition of ETP, whereas CT × 2 represents the anticoagulant concentration required to prolong clot time 2-fold. The values were obtained from the data shown in Fig. 4 as described in “Experimental Procedures.” Values represent means ± S.D. of triplicate determinations.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values without inhibitors</th>
<th>DX-9065a</th>
<th>Argatroban</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ETP IC₅₀</td>
<td>ETP IC₅₀</td>
<td></td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td></td>
</tr>
<tr>
<td>ETP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/3</td>
<td>2377 ± 39</td>
<td>&gt;6</td>
<td></td>
</tr>
<tr>
<td>1/10</td>
<td>1956 ± 74</td>
<td>4.12 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>1/30</td>
<td>1580 ± 83</td>
<td>2.07 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>1/100</td>
<td>746 ± 120</td>
<td>1.02 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>1/300</td>
<td>297 ± 86</td>
<td>1.08 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>Clot time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/3</td>
<td>1.82 ± 0.03</td>
<td>0.50 ± 0.06</td>
<td>1.24 ± 0.12</td>
</tr>
<tr>
<td>1/10</td>
<td>2.45 ± 0.02</td>
<td>0.51 ± 0.02</td>
<td>1.28 ± 0.13</td>
</tr>
<tr>
<td>1/30</td>
<td>3.35 ± 0.10</td>
<td>0.55 ± 0.02</td>
<td>1.26 ± 0.16</td>
</tr>
<tr>
<td>1/100</td>
<td>4.99 ± 0.08</td>
<td>0.61 ± 0.02</td>
<td>1.20 ± 0.10</td>
</tr>
<tr>
<td>1/300</td>
<td>7.13 ± 0.16</td>
<td>0.77 ± 0.19</td>
<td>0.99 ± 0.18</td>
</tr>
</tbody>
</table>

DISCUSSION

While thrombin generation initiated by extrinsic stimuli occurs in an explosive fashion during the propagation phase of blood coagulation, the thrombin concentration is very low during the initiation phase, i.e., before the propagation phase. The duration of the initiation phase normally coincides with the clot time of coagulation and, therefore, represents anticoagulant potencies if it is assessed in the presence of anticoagulants. The results of numerical simulation in the present study have predicted that FXa inhibitors and thrombin inhibitors might display comparable anticoagulant potencies as long as they are presumed to be competitive inhibitors with the same kinetic properties for interaction with their respective target enzymes. This was demonstrated experimentally by comparing DX-9065a and argatroban for their ability to prolong the clot time in human plasma (Fig. 3). It has been shown by these experiments that DX-9065a and argatroban are virtually equipotent in maintaining a subthreshold thrombin concentration during the initiation phase of extrinsically triggered plasma coagulation.

As predicted for the hypothetical inhibitors in the mathematical model, however, DX-9065a and argatroban exerted a differential influence on thrombin generation during the propagation phase of plasma coagulation. This was typically shown at the highest TF concentration examined, where DX-9065a reduced ETP much less effectively than did argatroban (Fig. 4A). The previous comparative studies on DX-9065a and other anticoagulants also demonstrated that DX-9065a was effective in prolonging clot time, but was less potent in reducing ETP than other anticoagulants (11, 12). Thus these results are in good agreement with those obtained at higher TF concentrations in the current study.

One explanation for the different abilities of the selective inhibitors to affect thrombin generation is that FXa inhibitors are able to inhibit de novo production of thrombin but not the ongoing actions of preformed thrombin. In contrast, thrombin inhibitors are able to inhibit not only the production of thrombin but also the actions of thrombin directly. The previous experimental studies, however, are not consistent with such a mechanism-based, straightforward rationalization. Hirudin and hirulog are known to be specific and direct inhibitors of thrombin, which delay thrombin peaks effectively during coagulation (12, 13), yet they do not reduce ETP as effectively as other anticoagulants (12) and display properties similar to those of DX-9065a. Although hirudin is a very potent inhibitor of thrombin, its tight but rather slow binding to thrombin (14) might somehow affect its overall anticoagulant properties in the plasma milieu. Furthermore, a recombinant tick anticoagulant peptide (11) and a pentasaccharide, SR-90107A (12), are two examples of FXa inhibitors reported to suppress ETPs more efficiently than DX-9065a. The reasons for these unpredictable observations are not clear at present. However, it should be pointed out that, for a given anticoagulant protease inhibitor, not only which enzyme is inhibited but also its precise mechanism of inhibition are important factors influencing its impact on the overall dynamic process during blood coagulation. Fortunately, both DX-9065a (1) and argatroban (2) are simple, competitive, reversible inhibitors, with which one can simply point out that, for a given anticoagulant protease inhibitor, not only which enzyme is inhibited but also its precise mechanism of inhibition are important factors influencing its impact on the overall dynamic process during blood coagulation. Fortunately, both DX-9065a (1) and argatroban (2) are simple, competitive, reversible inhibitors, with which one can simply postulate a rapid equilibrium for the dissociation-association interactions with their respective target enzymes. This might be a reason why numerical simulation models the dynamic effects of these compounds quite well. Thus, the findings presented in this report can be applied to other inhibitors of the same mechanism but only when they display similar kinetic properties.

The differential effects of the inhibitors in the simulation results are best perceived when thrombin activity curves are viewed with a proper time scale as shown in Fig. 2. If ETP, a time-integral of a thrombin activity curve, is calculated for an infinite time interval for a given set of numerical simulation data, the resultant ETP values approach invariable ones that are no longer dependent on inhibitor concentrations (data not shown). Thus, the differential effect presented in Fig. 2 should be regarded as an apparent one that does not necessarily rep-
resent the mathematical characteristics of the model. It is not known, however, whether the same explanation is applicable to the experimental results from the thrombin generation assays where ETP can be obtained only within a limited time scale.

Interestingly, the inhibitory effect of DX-9065a on ETP varied depending on the concentration of TF used to stimulate coagulation. The initiation of thrombin generation at the highest thromboplastin concentration resulted in up to more than a 5-fold increase in the ETP IC$_{50}$ value for DX-9065a compared with that at the lowest thromboplastin concentration (Table II). Such a TF-dependent effect was observed only when plasma was anticoagulated with DX-9065a. Argatroban did not show any TF-dependent effect on ETP IC$_{50}$ concentrations. The two compounds displayed only a limited difference in the ability to inhibit ETP at the lowest thromboplastin concentration (Fig. 4E and Table II). Moreover, no TF-dependent effect was observed with CT x 2 irrespective of whether plasma was anticoagulated with DX-9065a or argatroban (Table II).

In the absence of inhibitors, it was also shown that ETP increased with increasing TF concentrations (see the values without inhibitors in Table II). This observation may have some relevance to the observed TF-dependent effect of DX-9065a on ETPs. However the numerical simulation could not reproduce any of these TF-dependent effects on thrombin generation even when the initial concentration of TF:VIIa complex was varied over a wide range of concentrations (data not shown). The mechanism for the TF-dependent regulation is presently unknown. However, it seems reasonable to speculate that the observed TF-dependent effects might have some important roles in the regulation of blood coagulation.

REFERENCES
Studies on the Different Modes of Action of the Anticoagulant Protease Inhibitors DX-9065a and Argatroban: I. EFFECTS ON THROMBIN GENERATION
Hajime Nagashima

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