The Antithrombotic Efficacy of AT-1459, a Novel, Direct Thrombin Inhibitor, in Rat Models of Venous and Arterial Thrombosis

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Keywords

AT-1459, antithrombotic effect, bleeding time, clotting times

Summary

The antithrombotic efficacy of AT-1459, a novel, direct thrombin inhibitor (Ki = 4.9 nM) was evaluated in rat models of venous thrombosis combined with a bleeding time test and arterial thrombosis.

After drugs were given by i.v. bolus injection plus a continuous infusion, the ID₅₀ (a dose that exhibits 50% inhibition of thrombus formation over each vehicle group) values of AT-1459, argatroban, and dalteparin were 0.04 mg/kg plus 0.04 mg/kg/h, 0.1 mg/kg plus 0.4 mg/kg/h, and 13.0 IU/kg plus 26.0 IU/kg/h, respectively, in the venous thrombosis study. The BT₂ (a dose that causes 2-fold prolongation of bleeding time over each vehicle group) values of AT-1459, argatroban, and dalteparin were 0.9 mg/kg plus 0.9 mg/kg/h, 1.0 mg/kg plus 0.6 mg/kg/h, and 345.5 IU/kg plus 691.0 IU/kg/h in the rat tail transection model. The ratios of BT₂/ID₅₀ of AT-1459, argatroban, and dalteparin were 22.5, 10.0, and 26.6, respectively.

In a rat model of arterial thrombosis induced by topical FeCl₂ application, intravenous administration of AT-1459, argatroban, and dalteparin improved the vessel patency significantly (P < 0.01) at 0.6 mg/kg plus 0.6 mg/kg/h, 0.6 mg/kg/h plus 2.4 mg/kg/h, and 300 IU/kg plus 600 IU/kg/h, respectively.

The oral antithrombotic effect of AT-1459 lasted for 6 after administering 30 mg/kg and improved the vessel patency significantly 1 h after administering the same dose in venous and arterial thrombosis models, respectively, with a rapid onset of action. Warfarin also inhibited thrombus weight and improved the vessel patency significantly after oral administration of 0.3 mg/kg for three consecutive days in the same study. The antithrombotic and hemorrhagic effects of all drugs studied were correlated with plasma concentration or clotting times.

These results suggest that AT-1459 may be clinically useful as an orally available antithrombotic agent for the prevention of venous and arterial thrombosis.

Introduction

Venous thromboembolism, which includes deep vein thrombosis and pulmonary embolism, is a common disorder that can cause morbidity and even mortality among hospitalized patients if not adequately treated with anticoagulants (1). In addition, thrombus formation due to the disruption of atherosclerotic plaque in acute coronary syndromes causes platelet aggregation and the rapid development of thrombus in blood vessels which unfavorably reduces dynamic blood flow to produce more thrombus (2).

The important role that thrombin plays in the pathophysiology of thrombotic diseases as one of strong agonists for platelet activation and a key enzyme for formation of fibrin clot, has been emphasized by the potential that orally available direct inhibitors of this serine protease might exert a potent and long-term antithrombotic effect for preventing venous and arterial thrombosis (3).

Argatroban is a highly selective, potent, and direct inhibitor of free- and clot-bound thrombin (4) and can inhibit thrombin-induced platelet aggregation (5), with demonstrated antithrombotic effects in various animal models of thrombosis (6-8). Argatroban was also evaluated in clinical trials as an adjunct to thrombolytic therapy in patients with acute myocardial infarction (9). Recently, it was reported that a mixed micellar formulation of argatroban could be used as a potential antithrombotic agent for s.c. administration (10). However, the drug was launched as a parenterally available, but not an orally available formulation for the treatment of peripheral arterial occlusive disease and acute ischemic stroke (11).

Efforts at our research laboratories to identify an orally available direct thrombin inhibitor has led to the identification of AT-1459, a novel, direct thrombin inhibitor. In the present study, we describe the in vitro activity of AT-1459 and its antithrombotic effect in rat models of venous and arterial thrombosis.

Materials and Methods

Agents

AT-1459 (4-[(3S)-1-(2-((2S)-2-(6-amidino-1-ethylindol-2-yl)ethyl)pyrrolidinyl)-2-oxoethyl]-2-oxoazaperhydroepin-3-yl]amine) butanoic acid) was synthesized by C&C Research Laboratories (Fig. 1) (12). Dalteparin was...
purchased from Kissei Pharmaceutical Co., Ltd. (Fragmin® , Tokyo, Japan) and had a specific activity of 140 IU/mg (Factor Xa) and 62 IU/mg (thrombin). Argatroban was obtained from Daiichi Pharmaceutical Co., Ltd. (Slonnon®, Tokyo, Japan) and warfarin sodium was from Sigma Chemical Co. (St. Louis, MO, USA).

In in vitro studies, human α-thrombin, bovine trypsin, human activated protein C (aPC), human plasma kallikrein, human plasmin, and human urokinase (UK) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine factor Xa (FXa) was from New England Biolabs (Beverly, MA, USA). Human factor XIa (FXIa) was purchased from Enzyme Research Laboratories Inc. (South Bend, IN, USA). B-7632 (N-Benzoyl-Phe-Val-Arg-pNA) and V-0882 (D-Val-Leu-Lys-pNA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Chromozym®X and Chromozym®U were from Boehringer Mannheim (Mannheim, Germany). S-2366 was purchased from Daiichi Pure Chemical (Tokyo, Japan). For clotting time assays, all reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

In vivo studies, AT-1459 and warfarin were dissolved in saline (i. v. or p. o.), and argatroban extracted from Slonnon® was dissolved in a 12.5% solution of 99% EtOH diluted with saline. In control experiments there was no significant influence on the result when 12.5% EtOH was used as a vehicle.

**Enzyme Assay Using Chromogenic Substrates**

The inhibition of hydrolysis rate of each enzyme was assayed by measurement of absorbance at 405 nm at 25°C with a Microplate Reader (Molecular Devices Co., Menlo Park, CA, USA). 40 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl was used in all experiments. In some cases, 20 mM CaCl₂ was supplemented for studies of FXa, aPC, FXIa, UK or kallikrein.

Buffer containing each chromogenic substrate (160 μl), and inhibitors dissolved in 50% methanol (20 μl) were mixed in 96-well microplates. Reactions were initiated by the addition of 20 μl of enzyme solution containing 0.01% bovine serum albumin. Reagents and final concentrations are listed in Table 1.

**Animal**

Male Sprague-Dawley rats (330-430 g, Charles River Japan Inc., Yokohama, Japan) were used. The animals were anesthetized by an i. p. injection of 50 mg/kg pentobarbital Na, and tracheotomized. The right jugular vein was cannulated for i. v. administration of drug or vehicle.

**Table 1** Reagents and final concentrations in the determinations of Ki value for each enzyme

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme various units</th>
<th>Substrate μM</th>
<th>AT-1459 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>0.5 U/ml</td>
<td>B-7632 (50,70,100,200,300)</td>
<td>0-0.01</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1 U/ml</td>
<td>B-7632 (50,70,100,200,300)</td>
<td>0-0.01</td>
</tr>
<tr>
<td>FXa</td>
<td>20 ng/ml</td>
<td>Chromozym X (50,100,200,400)</td>
<td>0-1</td>
</tr>
<tr>
<td>FXIa</td>
<td>0.1 μg/ml</td>
<td>S-2366 (100,200,400,800)</td>
<td>0-1</td>
</tr>
<tr>
<td>aPC</td>
<td>0.1 μg/ml</td>
<td>S-2366 (100,200,400,800)</td>
<td>0-0.3</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>5 mU/ml</td>
<td>B-2133 (75,150,300,600)</td>
<td>0-1</td>
</tr>
<tr>
<td>Plasmin</td>
<td>4 mU/ml</td>
<td>V-0882 (50,100,200,400)</td>
<td>0-3</td>
</tr>
<tr>
<td>UK</td>
<td>25 mU/ml</td>
<td>Chromozym U (50,100,200,400)</td>
<td>0-10</td>
</tr>
</tbody>
</table>

**Venous Thrombosis Model Combined with Bleeding Time Test**

Our venous thrombosis model combined with a bleeding time test was produced by using a method described in our previous study (13). The inferior vena cava was carefully isolated and all branches from the vena cava were ligated. A partial stasis was made with a silk thread by tying a ligature around a 26 gauge 1/2 inch needle beneath the left renal vein and a caval sac of 1 cm length was produced by a clamp placed beneath the partial ligature. A separate 26 gauge 1/2 inch hypodermic needle was inserted into the inferior portion of the venous sac and hypotonic saline (0.225%) was infused at 2.5 ml/min for 15 s. The hypodermic needle was removed and the hole was sealed with cyanoacrylate cement. The needle used for a partial stasis was then freed from the ligature and the clamp was removed. The abdomen was closed with clips. After blood flow was maintained through the partial stasis for 20 min, the caval vein segment was removed from the rat using vascular clamps from the partial stasis to the iliac bifurcation. The exposed thrombus was extracted and weighed on the Sartorius balance (BP211D, Goettingen, Germany) after blotting each thrombus twice for 30 s on a tissue paper.

In the same rat, bleeding was induced by cutting the extremity of the tail (3 mm from the tip) with a surgical blade (Paragon No. 11) 3 min after vena cava sac construction. The bleeding time (BT) was determined by blotting the blood with a filter paper (Whatman No. 2) every 15 s until the bleeding stopped. BTs exceeding 15 min were noted as 15 min. The results were expressed as the ratio of BT in the drug-treated group over BT in the vehicle-treated group.

**Arterial Thrombosis Model**

Injury to the carotid artery was induced by using a modification method from the technique described by Kurz et al. (14). Briefly, the left carotid artery was carefully freed from the carotid sheath and a piece of parafilm (1 x 3 cm) was placed under the vessel. A Doppler flow probe (1 mm diameter, DBF-20A; Crystal Biotech, Hopkinton, MA, USA) was placed on the artery and connected to a Doppler velocimeter (HVPD-20; Crystal Biotech, Hopkinton, MA, USA) for recording on a polygraph (WS-682G, Nihon Kohden, Tokyo, Japan). A disc (2 x 3 mm) of filter paper (Whatman No. 1) was soaked in 40% solution of FeCl₃ (Merck, Darmstadt, Germany) and placed on top of the vessel downstream from the probe. The filter paper was removed after 10 min. Carotid patency was continuously monitored by the flowmeter for 60 min after FeCl₃ application. To evaluate the statistical significance of the drugs on vessel patency, patency status was expressed as follows: 1) persistent occlusion (PO):...
no reflow after occlusion, 2) reflow (RF): reflow or cyclic reflow after occlusion, 3) persistent patency (PP): persistent flow without occlusion after initial flow. After the end of the experiment, the segment of the vessel was isolated using vascular clamps. The exposed thrombus was extracted and weighed as described in our venous thrombosis study.

**Drugs**

Vehicle, dalteparin, argatroban, or AT-1459 were administered in i. v. doses as a bolus injection (2 ml/kg) plus a continuous infusion (5 ml/kg/h) from 15 min before vena cava sac construction until the end of the experiments, with established a steady-state plasma concentration level. The level in a steady state was evaluated by a HPLC analysis (AT-1459 and argatroban) or the prolongation of clotting times (dalteparin). The total infusion times were 35 and 75 min in the venous and arterial thrombosis study, respectively.

To evaluate the oral antithrombotic effect of AT-1459 or warfarin in rats overnight fasted, vehicle or AT-1459 were orally (2 ml/kg) administered using a gastric tube 0.5, 1, 2, 4, 6, 8, or 12 h before vena cava sac construction or 1 h before FeCl₃ application. Vehicle or warfarin (3 ml/kg) were also orally given once daily for three consecutive days and the last dose was administered 2 h before vena cava sac construction on the experimental day (the third day). The rats were anesthetized 10 min before preparing venous or arterial thrombosis and the surgical operation described above was carried out.

**Blood Sampling and Clotting Times**

0.9 ml of blood was collected from the right jugular vein with a 1 ml syringe containing 0.1 ml of 0.108 M sodium citrate solution. The samples were taken 1) prior to treatment with drugs, 2) 1 min after vena cava sac construction or FeCl₃ application, and 3) at the end of each experiment (at 35 or 75 min). Blood samples were also withdrawn at 0.5, 1, 2, 4, 6, 8, or 12 h after p. o. administration or 1 h after p. o. administration in the venous and arterial thrombosis study, respectively. Then, they were centrifugated at 15,000 rpm for 5 min at 4°C. The plasma was stored at -20°C until analyzed.

The active partial thromboplastin time (APTT), a clotting time (ECT), and prothrombin time (PT) were determined in a coagulometer (KC10; Amelung Co., Lemgo, Germany). In the APTT determination, 100 μl of plasma was incubated with 100 μl of prewarmed APTT reagent (A1926; Sigma Chemical Co.) at 37°C for 3 min. Coagulation was initiated by the addition of 100 μl of prewarmed 20 mM CaCl₂ solution (C7666; Sigma Chemical Co.). For the ECT assay, the time to clot was recorded by the addition of 75 μl of prewarmed echinoin (2 U/ml, E 0504; Sigma Chemical Co.) to 75 μl of prewarmed plasma (2 min at 37°C). In the PT determination, 100 μl of plasma was incubated for 2 min at 37°C. Coagulation was initiated by the addition of 200 μl of prewarmed PT reagent (T-9423; Sigma Chemical Co.). The clotting times exceeding 600 s were noted as 600 s.

The results were expressed as the ratio of the APTT, ECT, or PT after infusion (or after p. o. administration of AT-1459 or warfarin) over the baseline APTT, ECT, or PT before infusion (or after p. o. administration of vehicle).

**Plasma Concentration**

Plasma concentrations of AT-1459 and argatroban were determined by a reverse-phase HPLC (HP/1100, Hewlett-Packard, Waldbronn, Germany) using plasmas collected in both thrombosis studies. 200 μl of plasma was mixed with 1 ml of acetonitrile and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was dried using a rotary evaporator (MAXI dry plus, Heto, Allendorf, Denmark), and the plasma samples were reconstituted in 200 μl of mobile phase [5 mM PIC B5 (WAT084198, Waters Co., Milford, MA, USA); pH 1:1; pH 4.5]. 50 μl of samples were analyzed by HPLC with a column [UltraSphere-C8 (AT-1459) or UltraSphere-ODS (argatroban), 0.02 × 25 cm; Beckman, Fullerton, CA, USA]. The chromatographic condition of each drug was as follows: 1) AT-1459: The mobile phase consisted of 5 mM PIC B5 and acetonitrile, and methanol, programmed to be delivered at a flow-rate of 0.25 ml/min at the following gradient: 85:15 (initial); 60:40 at 25 min. AT-1459 was detected by monitoring its fluorescence (excitation = 300, emission = 453 nm). 2) Argatroban: The mobile phase consisted of 5 mM PIC B5, acetonitrile, and methanol, programmed to be delivered at a flow-rate of 0.25 ml/min at the following gradient: 93.5:2 (initial); 68:30:2 at 10 min; 8:90:2 at 25 min. Argatroban was detected by monitoring its UV-absorbance (252 nm).

**Statistical Analysis**

Drug effects were determined by one-way ANOVA, with the difference of mean thrombus weight between each drug and vehicle-treated groups detected by Dunnett’s test. Mann-Whitney rank sum test was used to evaluate the statistical significance of the drugs on vessel patency, time to occlusion (TT0), clotting times and BT. Computations were performed on an IBM computer using SigmaStat®2.0 (Jandel Co, San Rafael, CA, USA). All data are expressed as mean ± SEM (standard error of the mean) and P <0.05 was considered significant.

**Results**

**Enzyme Assay**

The inhibitory potency of AT-1459 against thrombin was 4.9 ± 0.4 nM. AT-1459 was classified as a competitive thrombin inhibitor as judged by a Lineweaver-Burk plot (data now shown). The selectivity of the compound over other serine proteases was shown in Table 2.

**Venous Thrombosis Study**

The mean thrombus weights in the vehicle-treated group were 9.1 ± 1.4 (saline) and 7.0 ± 0.7 mg (12.5% EtOH) per 100 g rat body weight in our venous thrombosis. All drugs after i. v. administration inhibited thrombus formation dose-dependently (Fig. 2A). The ID₅₀ of AT-1459 and argatroban were 0.04 mg/kg plus 0.04 mg/kg and 0.1 mg/kg plus 0.4 mg/kg/h, respectively, and plasma concentrations at those doses were 0.31 and 0.68 μM, respectively. The ID₅₀ of dalteparin was 13.0 IU/kg plus 26.0 IU/kg/h (Table 3).

Compared with the vehicle-treated group, the AT-1459-treated group resulted in a significant reduction of thrombus formation 0.5 to 6 (30 mg/kg) or 8 h (100 mg/kg) after oral administration, with a rapid onset of action. Its oral antithrombotic effect disappeared 8 (30 mg/kg) or 12 h (100 mg/kg) after administration (Fig. 4A). Warfarin inhibited thrombus formation significantly after oral administration of 0.3 to 1 mg/kg once daily for three consecutive days (Fig. 5).

**Table 2**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AT-1459</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>4.9 ± 0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>3.3 ± 0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>FXa</td>
<td>858.6 ± 181.8</td>
<td>175.2</td>
</tr>
<tr>
<td>FXIa</td>
<td>1165.0 ± 29.0</td>
<td>237.8</td>
</tr>
<tr>
<td>aPC</td>
<td>219.1 ± 0.3</td>
<td>44.7</td>
</tr>
<tr>
<td>KalliKrein</td>
<td>295.9 ± 0.6</td>
<td>60.4</td>
</tr>
<tr>
<td>Plasmin</td>
<td>1167.6 ± 348.0</td>
<td>238.3</td>
</tr>
<tr>
<td>UK</td>
<td>5875.2 ± 458.9</td>
<td>1199.0</td>
</tr>
</tbody>
</table>
Bleeding Time (BT)

Intravenous or oral administration of all drugs prolonged BT in a dose-dependent fashion (Fig. 2B). After i.v. administration, the BT$_2$ of AT-1459, argatroban, and dalteparin were 0.9 mg/kg plus 0.9 mg/kg/h, 1.0 mg/kg plus 4.0 mg/kg/h, and 345.5 IU/kg plus 691.0 IU/kg/h, respectively (Table 3). Compared with that in the vehicle treated-group, BT in the AT-1459-treated group was significantly prolonged 0.5 h (not more than 1 h) or 0.5 to 1 h (not more than 2 h) after orally administering 30 or 100 mg/kg (Fig. 4B). In preliminary experiments, there was no significant difference between the results in the BT test combined with the venous thrombosis model used in the present study and those in the BT test performed with the normal (non-instrumented) rats by the same method.

Arterial Thrombosis Study

Summarized in Table 4 are the data showing carotid artery patency, TTO, thrombus weight, the clotting times, and plasma concentrations after i.v. administration of dalteparin, AT-1459 or argatroban in a rat model of FeCl$_2$-induced arterial thrombosis. The patency status of the carotid artery in individual rats are also shown in Fig. 6. Stable occlusions due to arterial thrombosis were observed in all (6/6 or 7/7) rats of the vehicle group and the TTOs were 17.2 ± 0.7

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Various parameters of AT-1459, argatroban, and dalteparin in a venous thrombosis study. ID$<em>{50}$ or ID$</em>{30}$ means a dose causing 30 or 50% inhibition of thrombus formation. BT$_2$ means a dose causing 2-fold prolongation of BT over each vehicle group.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID$_{50}$ (mg/kg + mg/kg/hr)</th>
<th>ID$_{30}$ (mg/kg + mg/kg/hr)</th>
<th>BT$_2$ (IU/kg + IU/kg/hr)</th>
<th>BT$<em>2$/ID$</em>{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-1459</td>
<td>0.02 ± 0.02</td>
<td>0.04 ± 0.04</td>
<td>0.9 ± 0.9</td>
<td>22.5</td>
</tr>
<tr>
<td>Argatroban (mg/kg + mg/kg/hr)</td>
<td>0.06 ± 0.24</td>
<td>0.1 ± 0.13</td>
<td>1.0 ± 0.24</td>
<td>16.0</td>
</tr>
<tr>
<td>Dalteparin (IU/kg + IU/kg/hr)</td>
<td>6.4 ± 12.8</td>
<td>13.0 ± 26.0</td>
<td>345.5 ± 691.0</td>
<td>26.6</td>
</tr>
</tbody>
</table>
Dalteparin, AT-1459, and argatroban improved the vessel patency significantly (P < 0.01) at 300 IU/kg plus 600 IU/kg/h, 0.6 mg/kg plus 0.6 mg/kg/h, and 0.6 mg/kg plus 2.4 mg/kg/h, respectively. At those doses, stable blood flows were present in 3 of 7 rats in the dalteparin-treated group, in 6 of 8 rats in the AT-1459-treated group and in 5 of 8 rats in the argatroban-treated group, respectively, and the TTOs were 37.4 ± 8.0, 48.4 ± 6.6, and 48.3 ± 7.6 min, respectively. Compared with the vehicle-treated group (4.0 ± 0.7 mg in the saline-treated group and 3.9 ± 0.3 mg in the 12.5% EtOH-treated group), the dalteparin, AT-1459 or argatroban-treated groups produced a dose-dependent decrease of thrombus weight, with significant inhibition achieved at doses of 100 IU/kg plus 200 IU/kg/h (P < 0.01), 0.3 mg/kg plus 0.3 mg/kg/h (P < 0.05) and 0.3 mg/kg plus 1.2 mg/kg/h (P < 0.01), respectively (Table 4).

Table 5 represents the effects of AT-1459 and warfarin after oral administration. Stable occlusions due to arterial thrombosis were observed in all (7/7) rats of the vehicle group and the TTOs were 17.0 ± 1.0 min. Compared with the vehicle-treated group, the AT-1459-treated group resulted in the improvement of carotid artery patency and a significant reduction of thrombus formation 1 h after administering single 30 mg/kg p.o. Warfarin also improved the vessel patency, and inhibited thrombus formation significantly after oral administration of 0.3 mg/kg once daily for three consecutive days (Table 5). At those doses, stable blood flows were present in 5 of 7 rats in the AT-1459-treated group and in 6 of 7 rats in the warfarin-treated group, respectively, and the TTOs were 48.1 ± 7.7 and 54.6 ± 5.4 min, respectively. The patency status of the carotid artery in individual rats are also shown in Fig. 7.

Clotting Times

The baseline APTT (24-32 s), ECT (18-24 s), and PT (12-13 s) were similar in all groups of rats. All drugs after i.v. administration in our venous thrombosis study increased the clotting times dose-dependently. At ID_{50} and BT_{2}, the APTTs were prolonged 1.3 and 4.3, 1.2 and 2.4, and 1.7 and >12.0 times the baseline value for AT-1459, argatroban, and dalteparin, respectively, and the ECTs were increased 1.3 and >13.0, 1.7 and 5.0, and 1.0 and 1.2 times the baseline value for AT-1459, argatroban, and dalteparin, respectively.
The APTTs and ECTs in our arterial thrombosis study were prolonged 10.0 and 1.1, 3.4 and 8.4, and 2.1 and 5.3 times the baseline value for dalteparin, AT-1459, and argatroban at i. v. doses required to improve the vessel patency, respectively. Oral administration of AT-1459 and warfarin also prolonged the APTT, ECT or PT dose-dependently (Table 5).

### Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vessel patency</th>
<th>TTO (min)</th>
<th>Thrombus Weight (mg)</th>
<th>APTT ratio$^2$</th>
<th>ECT ratio$^2$</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n = 6)</td>
<td>6 0 0</td>
<td>17.2 ± 0.7</td>
<td>4.0 ± 0.7</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Dalteparin: 100 IU/kg + 200 IU/kg/hr (n = 7)</td>
<td>3 3 1</td>
<td>25.7 ± 6.0</td>
<td>2.4 ± 0.4**</td>
<td>2.6 ± 0.2**</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Dalteparin: 300 IU/kg + 600 IU/kg/hr (n = 7)$^{1,*}$</td>
<td>0 4 3</td>
<td>37.4 ± 8.0**</td>
<td>1.1 ± 0.1**</td>
<td>10.0 ± 1.2**</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Dalteparin: 1000 IU/kg + 2000 IU/kg/hr (n = 7)$^{1,*}$</td>
<td>0 0 7</td>
<td>60.0 ± 0.0**</td>
<td>0.3 ± 0.0**</td>
<td>11.2 ± 0.3**</td>
<td>1.3 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>AT-1459: 0.3 mg/kg + 0.3 mg/kg/hr (n = 9)</td>
<td>4 3 2</td>
<td>30.2 ± 5.8</td>
<td>2.6 ± 0.4*</td>
<td>2.2 ± 0.1**</td>
<td>5.0 ± 0.3**</td>
<td>1.94 ± 0.15</td>
</tr>
<tr>
<td>AT-1459: 0.6 mg/kg + 0.6 mg/kg/hr (n = 8)$^{1,*}$</td>
<td>1 1 6</td>
<td>48.4 ± 6.6**</td>
<td>1.4 ± 0.3**</td>
<td>3.4 ± 0.4**</td>
<td>8.4 ± 0.4**</td>
<td>3.78 ± 0.27</td>
</tr>
<tr>
<td>AT-1459: 1 mg/kg + 1 mg/kg/hr (n = 8)$^{1,*}$</td>
<td>0 1 7</td>
<td>55.5 ± 4.5**</td>
<td>1.0 ± 0.2**</td>
<td>4.8 ± 0.4**</td>
<td>15.0 ± 0.1**</td>
<td>7.01 ± 0.45</td>
</tr>
<tr>
<td>12.5% EtOH (n = 7)</td>
<td>7 0 0</td>
<td>17.7 ± 0.8</td>
<td>3.9 ± 0.3</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Argatroban: 0.3 mg/kg + 1.2 mg/kg/hr (n = 8)</td>
<td>4 3 1</td>
<td>28.6 ± 5.4</td>
<td>2.1 ± 0.4**</td>
<td>1.8 ± 0.2**</td>
<td>3.1 ± 0.1**</td>
<td>1.41 ± 0.13</td>
</tr>
<tr>
<td>Argatroban: 0.6 mg/kg + 2.4 mg/kg/hr (n = 8)$^{1,*}$</td>
<td>1 2 5</td>
<td>48.3 ± 7.6**</td>
<td>0.9 ± 0.2**</td>
<td>2.1 ± 0.1**</td>
<td>5.3 ± 0.3**</td>
<td>2.96 ± 0.31</td>
</tr>
<tr>
<td>Argatroban: 1 mg/kg + 4 mg/kg/hr (n = 8)$^{1,*}$</td>
<td>0 2 6</td>
<td>48.5 ± 7.4**</td>
<td>0.7 ± 0.2**</td>
<td>3.0 ± 0.3**</td>
<td>10.3 ± 0.2**</td>
<td>4.92 ± 0.61</td>
</tr>
</tbody>
</table>

The APTTs and ECTs in our arterial thrombosis study were prolonged 10.0 and 1.1, 3.4 and 8.4, and 2.1 and 5.3 times the baseline value for dalteparin, AT-1459, and argatroban at i. v. doses required to improve the vessel patency, respectively. Oral administration of AT-1459 and warfarin also prolonged the APTT, ECT or PT dose-dependently (Table 5).

### Plasma Concentration

Plasma AT-1459 and argatroban concentrations after i. v. administration were increased in a dose-dependent fashion, and correlated with their antithrombotic and hemorrhagic effects, and clotting times (Fig. 3 and Table 4).

### Discussion

In the present study, the antithrombotic effect of AT-1459 was compared to that of argatroban, dalteparin, or warfarin after i. v. or p.o.
administration in rat models of venous and arterial thrombosis. In our venous thrombosis model, combined with a tail transection bleeding time model in the same rat, we could evaluate simultaneously the anti-thrombotic and hemorrhagic effects of each drug in the same animal.

While AT-1459 at low i.v. doses potently inhibited thrombus formation, it prolonged BT significantly at the higher doses tested. When we compared the ratio of $\text{BT/iD}_{50}$ of AT-1459 (22.5) with that of argatroban (10.0), AT-1459 showed a more favorable antithrombotic/bleeding ratio than argatroban (Table 3). Additionally, AT-1459 exhibited a more shallow dose-response curve for efficacy and safety than argatroban. Dalteparin also exhibited a wide safety window (the ratio of $\text{BT/iD}_{50} = 26.6$) comparable to AT-1459 in our venous thrombosis study.

Animal models of venous thrombosis are frequently used to evaluate the antithrombotic effect and therapeutically relevant concentrations of novel anticoagulants. A main question in animal model studies is...
whether the outcomes from these studies can be applied to the clinical situation. Erickson et al. (15) reported that the concentration (0.42 ± 0.23 anti-Xa IU/ml) of dalteparin at 13 IU/kg i.v. plus 30 IU/kg/h that exhibited a potent antithrombotic effect (>90% inhibition of thrombus formation) in a rat model of venous thrombosis induced by the electrocauterization and ligation, was within the concentration range (0.3-0.8 anti-Xa IU/ml) recommended for prophylaxis of deep vein thrombosis in the clinical situation (16-17). It was also reported that 0.35-0.7 anti-Xa IU/ml of heparin in plasma almost corresponded to the APTT of 1.5 to 2.5 times the control value (18). The ID₉₀ of dalteparin was 13.0 IU/kg plus 26.0 IU/kg/h and the APTT ratio at that dose was 1.7 times the baseline value in the present study, suggesting that the ID₉₀ values of AT-1459, argatroban, and dalteparin might be a relevant dose for the treatment of deep vein thrombosis, although dose-finding results in animal models should be cautiously considered due to drug, model, and species differences. Furthermore, considering that a recommended intravenous dose of dalteparin in clinical practice is about 10 anti-Xa IU/kg/h (19), we may conclude that the clinically relevant dose of drugs studied will be approximately ID₉₀ lower than ID₉₀ in our venous thrombosis study (Table 3).

Dalteparin showed a potent antithrombotic effect without prolongation of BT in our venous thrombosis model. It was reported that compared to heparin administered i.v., low-molecular-weight heparins (LMWH), which give rise to a more favorable pharmacokinetic profile than heparin, were safe and effective in the treatment of patients with proximal deep vein thrombosis given twice daily s. c. (20). In addition, thanks to less intensive monitoring and the possibility of outpatient therapy, huge cost savings made the drug a good alternative for the treatment of venous thrombosis.

Acute coronary syndromes such as acute myocardial infarction and unstable angina are known to be due to thrombosis secondary to atheromatous plaque disruption (21). Particularly, the coronary thrombus is composed of a white head and a red tail, and the relative content of fibrin and platelet in the thrombus and the distribution of platelets are considered important since platelet-rich thrombus is less sensitive to thrombolysis. AT-1459 at 0.6 mg/kg i.v. plus 0.6 mg/kg/h and argatroban at 0.6 mg/kg i.v. plus 2.4 mg/kg/h were shown to improve the carotid artery patency significantly in our arterial thrombosis study. Although neither drugs at those doses caused significant prolongation of bleeding time, the effective doses of AT-1459 and argatroban were 10 and 5-fold as high as the ID₉₀ of both drugs in our venous thrombosis study, respectively. Owing to the inability of thrombin inhibitors to protect against platelet activation by other agonists like collagen, ADP and TXA₂ (22), and the high affinity of the platelet thrombin receptor for thrombin (23), it is known that relatively high concentrations of thrombin inhibitors are needed to inhibit thrombin-induced platelet aggregation than those of the drugs required to inhibit thrombin-mediated fibrin clot. An antidote may be needed to prevent patients with renal malfunction from bleeding due to overdoses. It was recently reported that Feiba™ has been shown to reverse the inhibition of thrombin generation by melagatran without affecting its antithrombotic effect in animal studies (24). It suggests that the bleeding risk of direct thrombin inhibitors in preventing arterial thrombosis may be mitigated by use of activated coagulation factors such as Feiba™; however this awaits confirmation in clinical trials.

In our arterial thrombosis study, the antithrombotic efficacy of AT-1459 was shown to be as potent as that of argatroban. On the other hand, dalteparin showed less potent antithrombotic efficacy than AT-1459 and argatroban. The limited potency of indirect thrombin inhibitors against arterial thrombosis can be accounted for the protection of thrombin from inactivation by heparin-antithrombin III complex by fibrin monomer and the inaccessibility of clot-bound thrombin to heparin (25, 26). Moreover, its clinical usefulness as an exclusively parenteral formulation may be less than orally available anticoagulants.

In the past decade pharmaceutical companies have been stimulated to develop orally active anticoagulants for long-term antithrombotic therapy. However, it was recently reported that the discontinuation of inogaturan treatment was followed by a reactivation of thrombin activity and ischemia (27). Rethrombosis, a rebound phenomenon due to the reactivation of the enzyme, is still an unsolved problem. In the present study, since we did not investigate the effect of AT-1459 on a rebound phenomenon, further investigation is thus required to evaluate whether the discontinuation of the drug is followed by arterial rethrombosis or restenosis after successful thrombolysis.

Warfarin, one of the vitamin K antagonists, is widely used as an oral anticoagulant. Despite its excellent bioavailability and predictable onset of action, warfarin has major limitations due to variability in dose responses among patients caused by food and drug interactions (28) and the therapeutic range of the drug is narrow. Thus, careful dosage adjustments are required to exhibit the optimal therapeutic effect without severe bleeding. Another limitation is that the anticoagulant effect of the drug is delayed for about four days. Additionally, its antithrombotic effect was accompanied with prolongation of BT in our venous thrombosis study, although the drug is safe if monitored with caution in human usage. On the other hand, AT-1459 inhibited thrombus formation significantly without severe prolongation of BT after oral administration of 30 mg/kg and its oral antithrombotic effect lasted for 6 h after administering that dose, with a rapid onset of action in our venous thrombosis study. Therefore, taking the limitations of warfarin into account, we can conclude that the major advantage of AT-1459 is its prompt onset of action (about 0.5 h) and moderate long-term activity after oral administration. Unlike indirect and direct thrombin inhibitors, however, warfarin at 0.3 mg/kg improved carotid artery patency significantly in the present study. This may be accounted for the pharmacological effect of warfarin that can inhibit hepatic biosynthesis of vitamin K-dependent coagulation factors including thrombin. AT-1459 improved carotid artery patency significantly 1 h after administering 30 mg/kg p. o., with a rapid onset of action unlike warfarin.

The plasma clotting time like APTT, PT, or ECT has been widely used as an index for monitoring the antithrombotic and hemorrhagic effects of thrombin inhibitors in animal experiments and clinical trials (29-30). In the present study, the ECT provided a more sensitive concentration-response curve to AT-1459 and argatroban as compared to the APTT at equi-effective antithrombotic and hemorrhagic concentrations. On the contrary, dalteparin showed a slight prolongation in the ECT at high doses (Table 4). The reason why ECT is not sensitive to high doses of dalteparin might be that unlike direct thrombin inhibitors, heparin-antithrombin III complex can not inhibit meizothrombin, which was generated by cleavage of scissile bonds in heterologous prothrombin by ecarin, and then autocalylate to α-thrombin (30).

AT-1459 inhibited thrombin strongly and competitively in a chro-
mogenic substrate assay and its in vitro activity against thrombin was >100 times more potent than against other serine proteases except for trypsin, aPC, and kallikrein. Until now, no information is available to guess what problems except bleeding risk can be caused due to the long-term administration of thrombin inhibitors with low or moderate selectivity in clinical situations. AT-1459 potently inhibited trypsin (Ki = 3.3 ± 0.5 nM) as well as thrombin. Although the enzyme does not normally occur in the blood and is not associated with haemostasis, the drug will encounter trypsin in the gut after oral administration. Further-
more, unlike argatroban, AT-1459 at BT2 (7.50 μM) or lower concentrations might inhibit other serine proteases significantly due to its moderate selectivity. Therefore, further studies are needed to clarify how much the drug could inhibit other enzymes at therapeutically relevant concentrations.

Taken together, AT-1459 exhibited potent antithrombotic effects after p.o. as well as i.v. administration in rat models of venous and arterial thrombosis. These results suggest that AT-1459 may be clinically useful as an orally available antithrombotic agent for the prevention of thrombotic diseases.

References


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