Antithrombin-Heparin Covalent Complex
A Possible Alternative to Heparin for Arterial Thrombosis Prevention

Anthony K.C. Chan, MBBS*; Janusz Rak, MD, PhD*; Leslie Berry, BSc*; Peng Liao, MD; Michal Vlasin, DVM, PhD; Jeffrey Weitz, MD; Petr Klement, DVM, PhD

Background—The anticoagulant effect of heparinoids is attributed to their cofactor activity for antithrombin (AT) and heparin cofactor II. In patients with thrombosis, however, thrombin is often protected from AT-dependent, heparin-mediated inactivation. The purpose of this study was to compare the properties of unfractionated/standard heparin (UFH/SH) and those of a novel covalent AT-heparin complex (ATH) in a rabbit arterial thrombosis prevention and bleeding model.

Methods and Results—Thrombosis in the distal aorta was triggered by vessel wall injury and critical stenosis. Blood flow in the damaged arterial segment was monitored with a flow probe placed distal to the constrictor. Rabbits were given doses of SH (62.5 to 187.5 IU · kg⁻¹ · 90 min⁻¹) or ATH (16 to 65 IU · kg⁻¹ · 90 min⁻¹). Cumulative blood loss from skin incisions was used to assess drug safety. The antithrombotic effects of ATH were greater than those of SH as measured by clot weight, blood flow, and vessel patency; eg, complete thrombus resolution was achieved with ATH (33 to 65 IU/kg), but not SH (125.0 to 187.5 IU/kg). At doses that produced equivalent vessel patency (50% to 60%), blood loss induced by ATH (60.2 µL) was 2.6-fold lower (P<0.05) than that induced by SH (154.6 µL).

Conclusions—In our experimental system, ATH was able to control arterial thrombosis more effectively than its SH precursor, without pronounced bleeding. (Circulation. 2002;106:261-265.)

Key Words: coronary disease ■ thrombosis ■ heparin ■ fibrin

Acute coronary syndromes result from acute thrombosis superimposed on a disrupted atherosclerotic plaque. Under such conditions, exposure of the subendothelial matrix leads to platelet adhesion, activation, and aggregation at the site of the vascular damage. At the same time, exposure of tissue factor present within the core of the atherosclerotic plaque promotes the assembly of clotting factors on the surface of activated platelets, resulting in a burst of thrombin generation. Eventually, thrombin is sequestered within the fibrin clot, where it is protected from inactivation by antithrombin (AT) and can trigger thrombus accretion.¹

Heparin and aspirin are cornerstones of therapy for acute coronary syndromes because of the crucial role of thrombin and platelets in the pathogenesis of these conditions. Both drugs, however, have significant limitations.² For example, heparin is antagonized by platelet factor 4 released from activated platelets. Perhaps the most important problem with heparin is that the heparin-AT complex has limited inhibitory activity against thrombin bound to fibrin (factor IIa) bound to fibrin and factor Xa bound to activated platelets trapped within thrombi.²³

In the liquid phase, binding between thrombin, heparin, and AT can occur freely in a combinatorial fashion, resulting in formation of inactive thrombin-antithrombin complexes. In contrast, the AT-heparin complex (ATH) has limited activity against thrombin bound to fibrin. This is because heparin also binds to both fibrin and thrombin and thereby facilitates accumulation of the latter in a “protected” active state.⁴ We hypothesized that if formation of the ATH preceded interaction with its thrombin target (free or bound), the entire pool of heparin would be involved in thrombin inhibition, resulting in a more efficient antithrombotic effect.⁴ We developed and tested a covalent ATH in which AT is prebound to heparin and demonstrated its potent anticoagulant activity in vitro and in vivo.⁵⁶ Because arterial thrombosis remains a major indication for anticoagulation with standard heparin (SH), an agent that exhibits several clinical limitations, we used a rabbit model of arterial thrombosis prevention to compare the properties of ATH and SH. We found that in this setting, ATH is more effective at restoration of blood flow without a parallel increase in bleeding.

Method

Materials
An unfractionated heparin/SH preparation (specific activity of 179 anti–factor Xa U/mg) was purchased from Sigma. AT was from

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Bayer. ATH was prepared as described previously and characterized by use of factor IIa and Xa inhibition assays. Gly-Pro-Arg-Pro-amide (GPRP)-NH$_2$, polybrene, and N-p-tosyl-Gly-Pro-Arg-p-nitroanilide (tGPR-pNA) (thrombin-directed synthetic substrate) were from Sigma.

**Rate of Thrombin Inhibition**

The rate of thrombin inhibition by either SH or ATH was determined discontinuously under pseudo-first-order conditions (molar ratio of enzyme to inhibitor was <10) by use of a method described previously. Briefly, human thrombin (Enzyme Research Laboratories) was incubated for 5 minutes in 96-well round-bottomed microtiter plate wells (Fisher) containing buffer (0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.01 mol/L GPRP-NH$_2$, and 0.6% polyethylene glycol 8000, pH 7.4) in either the absence or presence of 4000 nmol/L soluble fibrin monomer (prepared as described previously*). Incubation (SH plus 10-fold excess AT or ATH) was added to each well at different time intervals, followed by simultaneous determination of the inhibition reactions in all of the wells by addition of polybrene and tGPR-pNA substrate. The amount of thrombin remaining in each well was determined by measuring the rate of substrate hydrolysis in each case (A$_{405}$/min). Pseudo-first-order rate constants for the inhibition reactions were calculated according to Equation 1:

$$\frac{V_t}{V_0} = e^{-k_1 t}.$$  

where $V_0$ is enzyme activity at time 0 seconds, $V_t$ is enzyme activity at time $t$, and $k_1$ is the pseudo-first-order rate constant. Because of the high concentrations of inhibitor used in many experiments, reactions were carried out in the presence of tGPR-pNA, which acted as a competitive inhibitor. The concentrations of SH, AT, and ATH used in these experiments were in the 100 nmol/L range. In cases in which higher concentrations of AT were required, competition by the chromogenic substrate, the pseudo-first-order rate constant of inhibition was then calculated by use of Equation 2:

$$k_1 = \frac{k_{m}}{K_m} \times \left(1 + \frac{[S]}{K_m}\right),$$

where $k_{m}$ is the apparent pseudo-first-order rate constant, $[S]$ is the concentration of substrate, and $K_m$ is the Michaelis-Menten constant of thrombin for tGPR-pNA (14 mol/L). In a few experiments, pseudo-first-order rate constants were calculated by use of Equation 2 and compared with those calculated by use of Equation 1, where direct rate measurement in the absence of tGPR-pNA could be made with the same inhibitor concentrations. Equations 1 and 2 were carried out in the presence of tGPR-pNA, which acted as a competitive inhibitor.

**Animals**

Male New Zealand White rabbits (weight ~3.5 kg) were purchased from Charles River and housed for 7 days before the experiments. The experimental protocol was approved by the Animal Research Ethics Committee of McMaster University, Canada.

**Anesthesia**

Anesthesia was induced with a mixture of ketamine (50 mg/kg) and xylazine (2 mg/kg) given intramuscularly and maintained with a mixture of isoflurane (1% to 3%), oxygen (1.5 L/min), and nitrous oxide (0.5 L/min) through an endotracheal tube.

**Surgery**

We modified a previously published rabbit model system simulating the conditions of acute arterial thrombosis. Briefly, in brief, we induced thrombosis by creating a combination of critical stenosis, endothelial denudation, and vessel wall damage in a segment of the rabbit abdominal aorta. We monitored the parameters of blood flow and thrombus formation in the presence of vehicle, SH, or ATH. In addition, simultaneous evaluation of bleeding side effects was afforded by performing a “bleeding ear” assay on the same animal.

**End Points**

As an index of treatment efficacy, the weight of the mural clot was measured. Blood flow through the stenotic aorta and systemic blood pressure were monitored continuously during the course of each experiment. All measurements were conducted essentially as described previously.

**Statistics**

Data were expressed as mean ± SEM. ANOVA was performed to analyze data between treatment groups. Significant difference was set at a value of $P<0.05$. 

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*Figure 1. Arterial thrombosis prevention and ear bleeding rabbit model (see Methods and Reference 8).*

(Figure 1), as described previously. This “hybrid” model system permitted simultaneous evaluation of treatment efficacy and safety in an essentially continuous manner as well as monitoring of the systemic blood pressure and flow in different sections of the abdominal aorta.

Aortic stenosis was introduced as described previously, and the lumen reduction was maintained at >95% (as assessed by angiography). External damage of the aortic wall was induced by clamps proximal and distal to the stenosis. Blood pressure and aortic blood flow were recorded continuously; data were stored and analyzed later by use of the AT CODAS (Dataq Instruments Inc) data acquisition program.

**Treatment Groups**

The rabbits were randomized and treated as follows: Group 1, saline (n=18); intravenous (IV) bolus of saline 1 mL/rabbit followed by an IV infusion of 1 mL kg$^{-1}$ h$^{-1}$ for 90 minutes. Group 2, SH (n=6): IV bolus of 25 IU/kg; IV infusion of 25 IU kg$^{-1}$ h$^{-1}$ for 90 minutes (total of 62.5 IU/kg). Group 3, SH (n=6): IV bolus of 50 IU/kg; IV infusion of 50 IU kg$^{-1}$ h$^{-1}$ for 90 minutes (125 IU/kg). Group 4, SH (n=6): IV bolus of 75 IU/kg; IV infusion of 75 IU kg$^{-1}$ h$^{-1}$ for 90 minutes (187.5 IU/kg). Group 5, AT (n=6): IV bolus of 10 IU/kg; IV infusion of 4 IU kg$^{-1}$ h$^{-1}$ for 90 minutes (a total equivalent to 16 IU heparin/kg). Group 6, AT (n=6): IV bolus of 20 IU/kg; IV infusion of 9 IU kg$^{-1}$ h$^{-1}$ for 90 minutes (33 IU/kg). Group 7, AT (n=6): IV bolus of 40 IU/kg; IV infusion of 17 IU kg$^{-1}$ h$^{-1}$ for 90 minutes (65 IU/kg). A programmable Harvard apparatus (pump 44, model 4200-005, Harvard Apparatus Inc) was used for IV infusion of all treatments.

**Blood Collection**

Blood (0.9 mL) was collected into 0.1 mL of 3.8% sodium citrate at the time intervals indicated in Figure 1. Plasma was stored at −70°C for later analysis. One milliliter of citrated blood was also collected for calculation of the standard curve for bleeding experiments. Activated partial thromboplastin time, thrombin clotting time, and anti–factor Xa levels were determined by standard methods.
Results

Inhibition of Fibrin-Associated Thrombin Activity by ATH
Table 1 shows that SH is nearly 2 orders of magnitude less active against fibrin-associated thrombin than it is against the unbound enzyme. Thus, the second-order rate constants for heparin-catalyzed thrombin inhibition in the presence or absence of fibrin monomer were $6.54 \times 10^6 \pm 0.38 \times 10^6$ (mol/L)/min and $3.76 \times 10^6 \pm 0.10 \times 10^6$ (mol/L)/min, respectively. In contrast, covalent ATH complexes exhibited much more uniform (albeit not identical) activity against thrombin, both without and within the context of fibrin, a property that is reflected by the second-order rate constants of $2.26 \times 10^4 \pm 0.09 \times 10^4$ (mol/L)/min and $1.01 \times 10^4 \pm 0.13 \times 10^4$ (mol/L)/min, respectively. Thus, unlike SH, covalent ATH efficiently inhibits both free and fibrin-bound thrombin.

Differential Inhibition of Arterial Clot Formation in ATH- and SH-Treated Animals
We next compared the ability of SH or ATH to control arterial thrombosis in vivo. In our rabbit arterial thrombosis prevention model, vascular injury resulted in rapid clot formation and vessel occlusion in all control animals. Administration of either SH or ATH reproducibly inhibited this process in a dose-dependent manner, albeit with markedly differential efficiencies (Figure 2). SH in doses as high as 125 to 187 IU/kg did not completely block thrombosis, leaving measurable intraluminal fibrin deposits. In contrast, administration of comparable anticoagulant doses of ATH (Table 2) left the damaged arterial segments clot-free (Figure 2).

Assessment of the Efficacy/Safety Profile of ATH Versus SH in the Rabbit Model
Figure 3 shows cumulative blood flow curves obtained with either ATH or SH. It is apparent that lower doses of SH did not translate into any appreciable flow restoration in prothrombotic aortic segments. Even at the higher doses (125 IU/kg), the flow improved somewhat, but remained below 100 mL/90 minutes. In contrast, flow improvement was noted with all ATH doses. Even at doses of ATH equivalent to 33 to 65 IU heparin/kg, flow rates reached almost 200 mL/90 minutes (Figure 3). Also, whereas with SH a plateau was reached at 40 to 50 minutes, cumulative flow rates in the ATH groups (particularly at doses of 33 IU/kg and 65 IU/kg) continued to increase until the end of the experiment, suggesting a permanent inhibition of thrombosis.

To evaluate the safety-related aspects of ATH treatment, we carried out a series of coagulation assays and measured blood loss from standardized ear incisions in rabbits receiving the respective compounds. ATH produced a sharper and dose-dependent increase in the activated partial thromboplastin time ratio and anti–factor Xa activity, a change that was less pronounced in rabbits receiving SH. The differences between the highest doses of SH and ATH in terms of their ability to induce bleeding, however, were minimal and statistically insignificant (Table 2).

The interrelationship between cumulative blood loss and cumulative blood flow through the stenotic aorta with either ATH or SH administration is shown in Figure 4. It is apparent that at doses of ATH or SH associated with comparable preservation of blood flow (50 to 70 mL), the blood loss was more pronounced in rabbits treated with SH (130 to 160 mL) than with ATH (50 to 60 mL). Conversely, at doses that produced maximal cumulative blood loss (140 to 160 mL), blood flow in rabbits given ATH was 2- to 3-fold greater than in those treated with SH. Although plasma anti–factor Xa activities in the ATH group (65 IU/kg) were higher than those in the SH group (187.5 IU/kg) (Table 2), cumulative blood loss was essentially the same. Collectively, this analysis suggests that ATH has a more favorable benefit-to-risk profile than SH, at least in this model.

Discussion
Our data show that arterial thrombosis in our rabbit model can be effectively controlled by use of heparin covalently prebound to AT. Because of inherent heterogeneity of carbohydrate chains within various heparin preparations, considerable effort has been devoted to derivation of heparinoids with optimized structural properties. The key element of such preparations is thought to be the presence of the pentasaccharide sequence, which constitutes the binding site for endogenous AT. In addition, shorter sugar chains were produced, to reduce the nonspecific binding of heparin chains to plasma proteins and cells. These efforts have resulted in a series of low-molecular-weight heparins and synthetic pentasaccha-
ride\textsuperscript{3} directed at AT or heparin cofactor II.\textsuperscript{11} Although clinical evaluation of many of these preparations revealed an improved efficacy/safety profile, there is a continued need for further progress.\textsuperscript{2}

Direct thrombin inhibitors (eg, hirudin and Hirulog) are often considered as an alternative for heparinoids.\textsuperscript{8,12–16} Effective doses of hirudin, however, tend to suppress systemic hemostasis and increase the rate of bleeding. In addition, the absence of effective antidotes remains a reason for some concern.\textsuperscript{8,14,16}

Our report suggests that ATH may bypass some of the aforementioned limitations of SH. There are several reasons why this may be so. For example, it was suggested that factor Xa in the prothrombinase complex (prothrombin, factor Va, Ca\textsuperscript{2+}, phospholipid, factor Xa) is resistant to noncovalent mixtures of AT and heparin\textsuperscript{17} and may contribute to the procoagulant nature of thrombi.\textsuperscript{18,19} Both thrombin and factor Xa can change their accessibility to heparin and related inhibitors presented to them in the context of fibrin. Furthermore, excess heparin may have diminished (bimodal) anticoagulant activity. This is because both thrombin and AT must bind to a single heparin chain for the (thrombin) inhibition to take place.\textsuperscript{20} When excess heparin is present, however, the probability of separate thrombin/heparin and AT/heparin complex formation may increase to such an extent that assembly of the ternary (inhibitory) complex that constitutes thrombin/AT/heparin may occur at a low rate.\textsuperscript{4} In such a context, thrombin will appear refractory to SH treatment.\textsuperscript{21–26} ATH could overcome most of the aforementioned problems through several plausible mechanisms. Examples of those include (1) the nondissociable nature of the ATH complex, (2) the absence of separate heparin-fibrin interaction (hence, heparin could not bridge thrombin to fibrin), (3) the immediate availability of the active preassembled ATH inhibitory complex, (4) the absence of bimodal kinetics and “heparin excess” problems, (5) bypassing of the resistance of bound thrombin to noncovalent AT and heparin, and (6) the fact that ATH remains sensitive to protamine sulfate inactivation (our unpublished data).

The antithrombotic activity of ATH may also be influenced by potentiation of endogenous AT activity while remaining relatively independent of AT concentration in plasma.\textsuperscript{5} The synergistic interaction between ATH and AT, (ie, the “catalytic” activity of ATH) is afforded by the existence of 1 or more pentasaccharide units on the carbohydrate portion of the ATH molecule. A free pentasaccharide on the heparin chain in ATH (eg, the one not interacting with AT) could serve as a docking site for endogenous AT and thereby continuously contribute to its catalytic anticoagulant activity.\textsuperscript{5}

### Table 2. Effect of ATH on Blood Coagulation Parameters (aPTT, TCT, and Anti–Factor Xa Levels)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bleeding, μL</th>
<th>aPTT Ratio</th>
<th>TCT Ratio</th>
<th>Anti–Factor Xa U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>31.06±7.61*</td>
<td>0.99±0.30*</td>
<td>1.04±0.03*</td>
<td>0.00*</td>
</tr>
<tr>
<td>SH (187.5 IU/kg)</td>
<td>154.64±47.01</td>
<td>6.26±0.97</td>
<td>44.27±1.52</td>
<td>1.99±0.40</td>
</tr>
<tr>
<td>SH (125.0 IU/kg)</td>
<td>137.68±51.79 (NS)</td>
<td>2.57±0.36*</td>
<td>30.57±5.99*</td>
<td>1.71±0.37*</td>
</tr>
<tr>
<td>SH (62.5 IU/kg)</td>
<td>33.39±6.35*</td>
<td>2.43±0.40*</td>
<td>11.52±5.74*</td>
<td>0.97±0.22*</td>
</tr>
<tr>
<td>ATH (65.0 IU/kg)</td>
<td>161.71±53.65 (NS)</td>
<td>9.85±1.76*</td>
<td>41.20±2.56 (NS)</td>
<td>3.22±0.46 (NS)</td>
</tr>
<tr>
<td>ATH (33.0 IU/kg)</td>
<td>60.16±14.07*</td>
<td>3.13±0.28*</td>
<td>4.47±1.40*</td>
<td>1.68±0.11*</td>
</tr>
<tr>
<td>ATH (16.0 IU/kg)</td>
<td>50.88±13.83*</td>
<td>1.31±0.05*</td>
<td>1.15±0.06*</td>
<td>0.25±0.03*</td>
</tr>
</tbody>
</table>

*Values statistically significant vs highest dose of the reference compound (SH).
In conclusion, we report very encouraging antithrombotic properties of a novel AT-heparin complex. Further studies are necessary to understand in mechanistic detail which structural, biochemical, and pharmacological properties of ATH are essential for the characteristics we described. It also remains to be established where ATH may be most useful clinically. With respect to this, we have already tested ATH in models of arterial (shown here) and venous thrombosis.6 ATH could potentially be considered useful in conditions such as cardiopulmonary bypass, in which depletion of endogenous AT and extensive contact with prothrombotic extracorporal surfaces induce a systemic procoagulant state that is difficult to control with SH.27 We also foresee the application of ATH as a surface-bound anticoagulant for coating of artificial cardiovascular devices.28

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