

Modulation of Serine Proteases-Mediated Platelet Activation by Novel Direct Thrombin Inhibitors

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Abstract

Novel direct thrombin inhibitors (DTIs), such as bivalirudin, are replacing heparin in several clinical scenarios. In particular, DTIs are indicated for the treatment and thromboprophylaxis of patients with heparin-induced thrombocytopenia (HIT). In interventional cardiology, DTIs have several advantages over heparin, and offer a clinical benefit equivalent to that of a combination of heparin and anti-platelet agents. We hypothesize that this benefit results from the ability of DTIs to inhibit platelet activation by activated serine proteases. This study represents the development of a modified ^{14}C -serotonin release assay (SRA) to investigate the relative inhibitory effects of three DTIs (argatroban, bivalirudin and hirudin) on thrombin- and factor Xa-mediated ^{14}C -serotonin release (SR) in plasma-free systems. Washed platelets were isolated from blood of healthy volunteers. The ^{14}C -SRA test was similar to that used to detect heparin-PF4 antibody-mediated platelet activation, except that it was used to evaluate the ability of DTIs to modulate protease-induced SR responses. The inhibitory effects of DTIs were determined at protease concentrations that induced $\geq 50\%$ SR. Serine proteases induced SR from platelets in a concentration-dependent manner. Human thrombin was found to be more potent

than bovine thrombin (2-3 times for 50-80% SR). Bovine factor Xa (≥ 0.2 nKat/ml) produced a comparable (50-80%) SR response. All three DTIs effectively blocked serine protease-mediated platelet activation in a concentration-dependent manner. The optimum inhibitory concentrations of bivalirudin on SR was ~ 100 nM for human thrombin and bovine factor Xa and almost double for bovine thrombin; well below plasma concentrations necessary for effective anticoagulation for percutaneous coronary interventions. Wide variations in the inhibitory effects of each DTI on thrombin- and factor Xa-mediated platelet activation were noted, which was partly dependent on the donor platelets and stability of the proteases/inhibitors. It is concluded that DTIs can directly inhibit serine proteases-mediated platelet activation responses.

Keywords: Hirudin, thrombin, factor Xa, serine proteases, thrombogenesis.

Introduction

Serine proteases, such as thrombins and Factor Xa, play key role(s) in thrombogenesis and are capable of activating platelets through various mechanisms. Thrombin is not only a catalyst in the conversion of soluble fibrinogen into an insoluble fibrin clot, but is also an extremely potent platelet activator. Thrombin mediates platelet agonist effect through a unique and specific proteolysis of cell surface receptor known as PARs (protease-activated receptors). Two of the known PARs (namely, the PAR-1 and PAR-2) are expressed by human platelets.¹ The PAR-1 has been designated as having a higher affinity for thrombin than PAR-4.² Recently, it has also been shown that thrombin binds to the platelet glycoprotein (GP) Ib/IX/V complex, which supports a role for GPIb ∞ in thrombin-induced platelet activation/aggregation.³

Despite a remarkable therapeutic spectrum, the use of heparins is known to have medical complications such as bleeding and the onset

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of a potentially catastrophic syndrome known as heparin-induced thrombocytopenia (HIT) associated with or without thrombosis.^{4,5} Heparin as an indirect thrombin inhibitor increases the ability of antithrombin III (AT) to neutralize thrombin and other serine proteases of the coagulation cascade. Platelets, vascular surfaces, plasma proteins, and fibrin are all key factors in this anticoagulant effect of heparin. Heparin's indirect mechanistic limitations are: i) its inability to inactivate thrombin bound to fibrin, therefore, protecting it from inactivation by the heparin-AT complex,⁶ and ii) its inability to inactivate platelet bound factor Xa, therefore, sheltering it from the heparin-AT complex while secreting platelet factor 4 (PF4), a heparin neutralizing protein.⁷ These limitations make heparin a competitive inhibitor for circulating non-bound thrombin, but unfortunately leave room for a desirable alternative for clot-bound thrombin anticoagulants.^{8,9}

Because of their pharmacokinetic and biological advantages, direct thrombin inhibitors (DTIs), such as argatroban, bivalirudin and hirudin, have recently been developed as a heparin substitute for various clinical indications.¹⁰⁻¹² In particular, these agents are widely used as the alternate anticoagulant management of heparin-compromised patients (e.g., HIT, where massive thrombin generation occurs in symptomatic patients), requiring therapeutic or interventional anticoagulation.^{13,14} The DTIs have shown a clear advantage due to their ability to inhibit both clot-bound and circulating thrombin. These agents prevent thrombin from interacting with its substrates by binding to either or both the active site and the exosite-1. These two sites are responsible for the modulation of thrombin-substrate interactions. The active site is responsible for cleaving the scissile bond, while the proper orientation of the substrate is determined by exosite-1.¹⁵

Thus, DTIs have several advantages over heparin and offer a clinical benefit equivalent to that of a combination of heparin and anti-platelet agents. We hypothesize that this

benefit results from the ability of DTIs to modulate platelet activation by activated serine proteases. In this study, a modified ¹⁴C-serotonin release assay (SRA) was developed to study the relative inhibitory effects of various newly developed DTIs (in plasma-free system), on the platelet activation induced by serine proteases (thrombin and factor Xa).

Materials and Methods

Materials

The materials used in this study and their sources are given in parentheses: human thrombin and bovine factor Xa (Diagnostica Stago, Gennevilliers, France), bovine thrombin (Pacific Hemostasis, Middletown, VA), 5-hydroxy-¹⁴C-tryptamine creatinine sulfate (Amersham, Piscataway, NJ), argatroban (GlaxoSmithKline, Philadelphia, PA), bivalirudin (The Medicines Company, Parsippany, NJ), hirudin, apyrase Grade III from potato, and scintillation fluid/universal LSC cocktail (SigmaAldrich, St. Louis, MO). Other reagents used in this study were of analytical grade and from the best commercial sources available.

Preparation of Washed Platelets

The platelet isolation procedure was similar to that described earlier.¹⁶ Briefly, human whole blood (WB) from normal healthy volunteers was collected by venipuncture into acid-citrate-dextrose (ACD) anticoagulant, pH 4.5 (1 part ACD: 5 parts WB). The first 3 ml of WB were discarded to avoid any pre-activation of platelets. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 300 *g* for 15 min at room temperature. The PRP was incubated with ¹⁴C-serotonin 0.1 μ Ci/ μ l (2 μ l of ¹⁴C-serotonin for per ml PRP, having specific activity of 2.07 GBq/mmol 56.0 mCi/mmol) for 45 min at 37°C. Centrifugation of the ¹⁴C-labeled-PRP at 600 *g* for 10 min at room temperature yielded a platelet pellet. Platelets were washed with 10 ml calcium-albumin-free (CAF) buffer, pH 6.2, containing apyrase as described above. Finally, the pellet was resuspended in albumin-free-Tyrode's (AFT) buffer, pH 7.4, at a platelet concentration of 250,000 - 300,000/ μ l for the ¹⁴C-SRA experiments.

¹⁴C-Serotonin Release Assay (SRA)

The ¹⁴C-SRA test system used in this study was similar to that utilized in the heparin-PF4 (HIT) antibody-mediated platelet activation responses,¹⁷ except that instead of patients' plasma and exogenous heparin, we evaluated the effects of serine proteases to cause platelet activation (serotonin release as an end-point index) under different experimental conditions, and subsequently determined the modulation of platelet activation by various DTIs. The first assay condition evaluated the effect of varying concentrations of serine proteases (bovine and human thrombins, and bovine factor Xa) to achieve the optimal platelet activation responses. Once the optimal serine protease concentration was established, we investigated the modulating effects of DTIs (such as argatroban, bivalirudin, and hirudin) on the serine protease-mediated platelet activation responses.

Briefly, the serine proteases (10 µl in saline, at varying concentrations) were incubated in a round-bottom 96-well plate with ¹⁴C-serotonin-labeled washed platelets (70 µl) while shaking gently for 60 min at room temperature. The platelet activation process was terminated by the addition of 100 µl of EDTA (4% solution in saline). The content was centrifuged at 1,600 *g* for 5 min at room temperature and 50 µl of the supernatant was transferred to a scintillation vial, pre-filled with 2.5 ml of scintillation fluid (universal LSC cocktail). The radioactivity of each sample was determined on a β-counter connected with a printer (Wallac, Inc., Gaithersburg, MD). The AFT buffer and 10% Triton X-100 solution (30 µl each) were run simultaneously, which served as 0% and 100% controls of the serotonin release responses, respectively.

To determine the effect of various DTI's concentration necessary to inhibit the serine proteases-mediated serotonin release responses, we utilized the similar approach as described above, except that varying concentrations of DTIs (10 µl in saline) were incubated with washed-radiolabeled platelets (70 µl) and proteases (10 µl at a pre-determined concen-

tration where $\geq 50\%$ serotonin release response was achieved).

Data Analyses

The following formula was used to calculate the percent serotonin release: % serotonin release is equal to the release of the test sample minus the background (the AFT buffer response), divided by the total radioactivity (the Triton X-100 response) minus the background (AFT), multiplied by 100. A \pm SEM (standard error mean) was also calculated to account for the certainty of sample means among the data obtained from multiple donors' platelets. Statistical significance was declared at *p* value <0.05.

Results

The optimal concentration of each serine protease to produce the maximum platelet activation (percent serotonin release) was determined by taking appropriate concentrations of the proteases. *Fig. (1A)* displays the percent serotonin release response upon activation of platelets by varying concentrations of human thrombin. Human thrombin at a concentration of 0.15 U/ml resulted in an average of $80 \pm 3.9\%$ serotonin release while 0.03 U/ml of human thrombin gave an average of $50 \pm 7.8\%$ serotonin release. Concentrations < 0.015 U/ml of human thrombin were found to produce < 40% serotonin release, which was considered to be less than optimal. The fixed (optimum) concentration for human thrombin to achieve a range of 50-80% serotonin release from activated platelets, was therefore, set at 0.1 U/ml.

Fig. (1B) shows the concentration-dependent response on platelet activation by bovine thrombin. As can be seen, an optimal concentration range of 0.08-0.4 U/ml of bovine thrombin was sufficient to cause a platelet activation response ($79 \pm 3.5\%$ serotonin release). The maximum serotonin release of $91 \pm 3.3\%$ was, however, found to be achieved at a concentration of nearly 1.0 U/ml of bovine thrombin. There was a steep decline in serotonin release response when bovine thrombin was used at concentration below 0.1 U/ml (only 4-30% release).

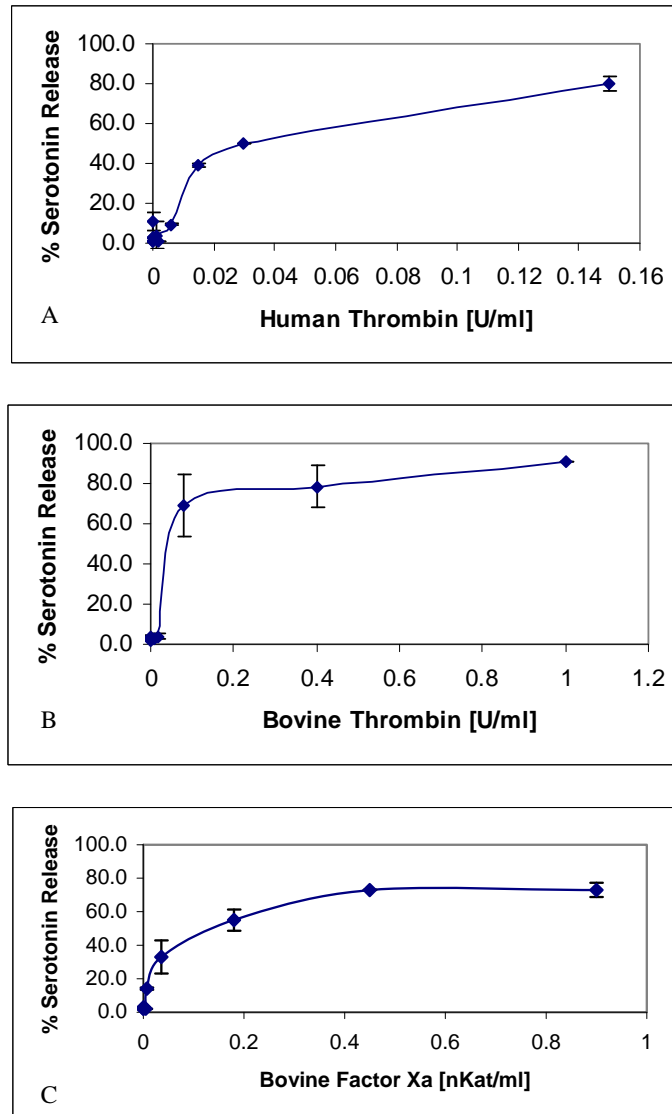


Fig. (1). Concentration-dependence of the effect of serine proteases on ¹⁴C-serotonin release from washed human platelets. The assay was employed to determine the optimal concentration of serine protease required to produce at least 50-80% ¹⁴C-serotonin release response. Each data point in the panel: (A) human thrombin, (B) bovine thrombin, and (C) bovine factor Xa, represents the average results obtained from different donors' platelets (n=3) and the data are reported as mean ± SEM of the percent ¹⁴C-serotonin release upon platelet activation.

Data obtained from the bovine factor Xa-mediated serotonin release response is shown in Fig. (1C). Clearly, about 0.5 nKat/ml of bovine factor Xa was found to be sufficient to produce 73 ± 3% serotonin release. Although, bovine factor Xa at nearly 0.2 nKat/ml resulted in about 55 ± 4.2% serotonin release but any further lowering of factor Xa concentration resulted in the sharp decline of platelet activation responses.

The inhibitory effects of various DTIs were determined at the fixed proteases concentrations that induced >50% serotonin release (usually in the range of 50-80% release, as optimized above). Fig. (2) shows the results obtained on the concentration-dependence of argatroban to inhibit various protease-mediated platelet activation responses. Bovine thrombin-mediated platelet activation appeared to be most sensitive to argatroban

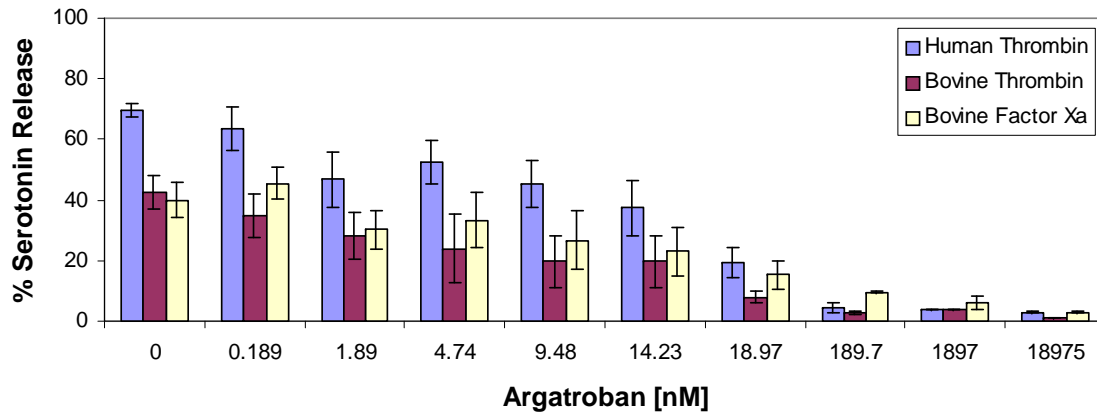


Fig. (2). Effect of argatroban concentration to modulate the serine proteases-mediated platelet activation responses (^{14}C -serotonin release). Washed human platelets were activated at fixed (optimum) concentration of human thrombin (0.1 U/ml), bovine thrombin (0.1 U/ml), and bovine factor Xa (0.5 nKat/ml). High percent of ^{14}C -serotonin release (>50%) can be seen at lower concentrations of argatroban, which is greatly inhibited at higher concentrations of the drug. Each data point represents the average results obtained from different donors' platelets ($n=5$) and the data are reported as mean \pm SEM of the percent ^{14}C -serotonin release upon platelet activation.

as only 5-10 nM (which is equivalent to 2.5 – 5.0 ng/ml) of argatroban was sufficient to inhibit significantly ($< 25 \pm 11\%$ serotonin release), whereas human thrombin- and bovine factor Xa-mediated platelet activation required relatively much higher concentrations of argatroban (at least 19 nM) to achieve a comparable inhibitory response. While platelet activation modulation by argatroban varied for hu-

man thrombin, bovine thrombin, and bovine factor Xa, all serine proteases-mediated activations were almost completely inhibited at 190 nM of argatroban (resulting only in $< 10 \pm 1\%$ serotonin release).

Fig. (3) shows the effect of serine proteases-mediated platelet activation response and its modulation by bivalirudin. Bivalirudin showed a very strong concentration-dependence on

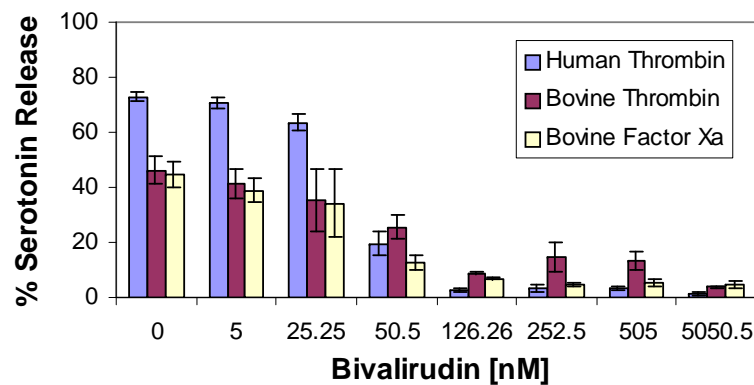


Fig. (3). Effect of bivalirudin concentration to modulate the serine proteases-mediated platelet activation responses (^{14}C -serotonin release). Washed human platelets were activated at fixed (optimum) concentration of human thrombin (0.1 U/ml), bovine thrombin (0.1 U/ml), and bovine factor Xa (0.5 nKat/ml). High percent of ^{14}C -serotonin release (> 50%) can be seen at lower concentrations of bivalirudin, which is greatly inhibited at higher concentrations of the drug. Each data point represents the average results obtained from different donors' platelets ($n=6$) and the data are reported as mean \pm SEM of the percent ^{14}C -serotonin release upon platelet activation.

the inhibition of proteases-mediated platelet activation responses. The optimal inhibitory concentration of bivalirudin on serotonin release was determined to be ~100 nM, which is equivalent to < 0.1 µg/ml (for human thrombin and bovine factor Xa). However, for bovine thrombin-mediated platelet activation, a relatively higher concentration of bivalirudin was required to achieve such response. These concentrations of bivalirudin were still well below the plasma levels of the drug necessary for effective anticoagulation during percutaneous coronary interventions (PCI).

Similarly, we tested the comparative inhibitory effects of hirudin (another potent and widely used DTI) on the proteases-mediated platelet activation responses. Fig. (4) shows that hirudin also caused a concentration-dependent inhibition on all the proteases-mediated platelet activation responses. Again, as low as 14 nM (equivalent to ~0.1 µg/ml) of hirudin was sufficient to modulate the proteases-mediated serotonin release by ≤ 10%.

Discussion

In recent years, several new DTIs have emerged as alternate anticoagulants to heparin in various clinical situations including treatment and thromboprophylaxis of patients with HIT and prevention of acute coronary events and thrombosis.¹⁰⁻¹⁴ In this study, we compared the thrombin inhibitory effects of some novel DTIs on specific serine protease-mediated platelet activation (serotonin release) responses in plasma-free systems. Our results clearly indicate that despite some variable inhibitory effects of the DTIs (presumably due to their differential biochemical properties and mechanism of action), all these agents, particularly bivalirudin, are capable of modulating the thrombin- and factor Xa-mediated platelet activation responses, at well below the plasma concentrations necessary for effective anticoagulation in cardiovascular indications, such as PCI.

Serine proteases are known to play central role(s) in the coagulation cascade and have

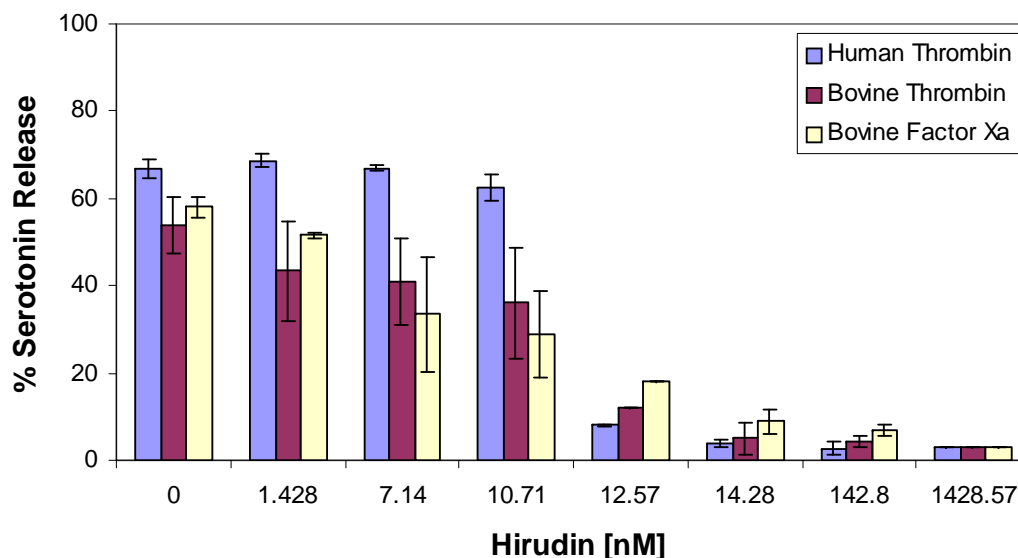


Fig. (4). Effect of hirudin concentration to modulate the serine proteases-mediated platelet activation responses (¹⁴C-serotonin release). Washed human platelets were activated at fixed (optimum) concentration of human thrombin (0.1 U/ml), bovine thrombin (0.1 U/ml), and bovine factor Xa (0.5 nKat/ml). High percent of ¹⁴C-serotonin release (> 50%) can be seen at lower concentrations of hirudin, which is greatly inhibited at higher concentrations of the drug. Each data point represents the average results obtained from different donors' platelets (n=3) and the data are reported as mean ± SEM of the percent ¹⁴C-serotonin release upon platelet activation.

long been known to participate in thrombogenesis and cause platelet activation through direct or indirect mechanisms.¹⁻³ In this investigation, we implemented a novel approach by selecting three widely explored serine proteases (namely, human and bovine thrombins and bovine factor Xa) to evaluate their relative ability to activate washed human platelets and thus specifically quantified one of the final released products upon platelet activation (such as ¹⁴C-serotonin). The modulatory effects of DTIs on these radio-labeled platelet serotonin release responses were systematically investigated.

Table 1 compares some of the biochemical and clinicopharmacological properties of the three DTIs with that of unfractionated and low-molecular-weight heparins. The discussion below focuses on additional characteristics of the three DTIs that we used in this investigation that are clinically relevant and on its relationship with experimental observations.

Argatroban (Novastan[®]), one of the first synthetic DTIs, is a small molecular weight (526.66 D) compound derived from *L*-arginine with reported *K_i* values of 19-39 nM.¹⁸ This reversible thrombin inhibitor is readily metabolized in the liver and has a molecular formula of C₂₃H₃₆N₆O₅S·H₂O. Thrombin inhibition occurs by

directly blocking the active site on thrombin.¹⁹ The argatroban-thrombin complex has two hydrophobic side chains that create a U-shaped configuration connecting with the S2 and the aryl-binding pocket, both hydrophobic domains of thrombin.^{20,21} The highly specific inhibition of thrombin is allowed by a Tyr-Pro-Pro-Trp loop found in the pocket,²² which allows argatroban to have a highly selective nature towards thrombin in comparison to other drugs. Because of its solubility, this drug is normally administered via an intravenous bolus followed by infusion. The advantages of this agent lie in its small molecular weight and the ability to directly interact with the active site (*Table 1*). It is also notable that argatroban lacks the generation of any clinically significant antibodies.²³ As shown in *Fig. (2)*, although argatroban had the most potent inhibitory effect on bovine thrombin-mediated platelet activation, human thrombin- and bovine factor Xa-mediated serotonin release response was also modulated by this agent at a relatively lower and concentration-dependent manner.

Bivalirudin (Angiomax[®]), a novel specific and reversible DTI, which is a recombinant protein based on hirudin, and composed of a 20 amino acid peptide analogue of the carboxy-terminal region of hirudin, linked *via* four

Table 1. Comparison of some of the molecular characteristic properties of direct thrombin inhibitors with unfractionated heparin and low-molecular-weight heparins

Agents	Size (MW)	Mechanism of Action	Reversibility	Metabolized	Advantages
Argatroban	527 D	Direct: Active Site	Reversible	Liver	Small Molecular Weight
Bivalirudin	22 aa	Direct: Active Site and Exosite	Reversible	Liver, and 20% Renal	Strength plus Reversibility
Hirudin	65 aa	Direct: Active Site and Exosite	Non-reversible	Kidney	Strength of Inhibition
UFH	12,000 – 15,000 D	Indirect	Reversible	20-50% Excreted Unchanged, Some Hepatic Metabolism	Non-anticoagulant Effects
LMWH	2,000 – 8,000 D	Indirect	Reversible	20-50% Excreted Unchanged, Some Hepatic Metabolism	Less Monitoring and Immunogenic Response

Size and/or molecular weight (MW) is measured either in Daltons (D) or amino acids (aa) peptide sequence length. The non-anticoagulant effects for heparins [both unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH)] include the release of cytokines, anti-inflammatory mediators, tissue factor, and adhesion molecules. The LMWHs have the same benefits as UFH, while it has less of an immunogenic response such as in heparin-induced thrombocytopenia.

Gly residues to D-Phe-Pro-Arg-Pro, which interact with the active thrombin site (both circulating and clot-bound thrombin). Characteristically, bivalirudin directly inhibits thrombin by binding both to the catalytic site and to the anion-binding exosite (derived from residues 53-64 of hirudin), thereby blocking serine proteases-mediated platelet activation and/or aggregation. Bivalirudin's bivalent mechanism of action occurs in a ratio of 1:1 and involves thrombin's cleavage of fibrinogen and its activation of factors V and VIII.^{15,24,25} The clearance of bivalirudin is thought to be mainly metabolic, which may include clearance by the liver or proteolysis at other sites including the vascular compartment. Only 20% of bivalirudin is removed renally.²⁶ Bivalirudin has been shown to have clinical advantages such as reducing the risk of ischemic complications and the reduced risk of bleeding.^{27,28} To date, no confirmatory report exists about the immunogenic response in patients treated with bivalirudin. As shown in *Fig. (3)*, this experimental observation clearly demonstrates that bivalirudin is a highly potent inhibitor of serine proteases-mediated platelet activation at a much lower concentration usually required for anticoagulation during PCI.

Hirudin (Refludan[®]), another potent and high molecular weight DTI obtained from *Hirudo medicinalis*, a medicinal leech located in the salivary glands, is a 65 amino acid compound and is administered as a bolus.²⁹ Hirudin can be used in two forms, both native and recombinant. While the two forms may differ characteristically, their clinical advantages of being a DTI are by and large the same. It is an extremely potent DTI in that it forms an irreversible complex with thrombin in a stoichiometric ratio of 1:1. This strong bond occurs on multiple sites and eliminates the need for circulating antithrombin.³⁰ Hirudin's mechanism of action is *via* both the active site and the exosite-1. This inhibition of thrombin occurs by the amino-terminal domain inhibiting the active site, while exosite-1 binds to the acidic carboxy-terminal domain.^{7,8} Hirudin is metabolized in the kidneys, which poses a limitation for this agent,

and because of this limitation, patients being treated for impaired renal function can not use hirudin.^{15,29} Furthermore, recent reports suggest that >40% of hirudin-treated HIT patients develop drug-specific antibodies that enhance/suppress the anticoagulant activity of hirudin.³¹⁻³³ Despite these limitations, reports have shown some clinical advantages of hirudin over heparin, i.e., reduction of the risk of death or myocardial infarction at 24 and 48 h post-treatment in GUSTO IIb study,³⁴ and in unstable angina patients.⁷ Further research has indicated that hirudin is more effective in preventing new ischaemic events, revascularization procedures, and new myocardial infarction than heparin as in OASIS studies.^{35,36} In our laboratory studies, we indeed found that like other DTIs, hirudin is also an equally good inhibitor modulating the serine proteases-mediated serotonin release responses in a concentration-dependent manner.

While all the DTIs - just like any other anti-thrombotic, antiplatelet, or thrombolytic agents - have some advantages and disadvantages over heparin in specific clinical settings, particularly in cardiovascular indications. Bivalirudin and related DTIs investigated in this study clearly show that they could effectively modulate the serine proteases-mediated platelet activation at concentrations well below the plasma levels generally required for effective anticoagulation in cardiovascular interventions. Such effects may be of particular benefits to the management of patients with HIT as well as other patients (e.g., with diabetes, hypertension, inflammation and shock), where platelet activation is often associated with massive thrombin generation and hypercoagulable state.

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