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Inhibition of thrombin: relevance to anti-thrombosis strategy

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1. ABSTRACT

Thromboembolism is a common cause of death and disability. Heparin or warfarin, the current standard management for thromboembolism may cause serious bleeding complications. Thrombin is the key enzyme of coagulation. Hirudin, the most potent natural thrombin-specific inhibitor, was first isolated from leech salivary fluid. Synthetic thrombin-specific inhibitors are rationally designed based on the knowledge on the structures of the activate site of thrombin. Thrombin-specific inhibitors are the current best choice for the treatment of heparin-induced thrombocytopenia (HIT). Recombinant hirudins (such as desirudin) were also approved for the prevention of thrombosis after hip or knee surgery. Bivalirudin (hirulog-1 or Angiomax), in adjunct to aspirin, was approved for prevention of thrombosis in patients with unstable angina following angioplasty. Argatroban has been used for the treatment of HIT, peripheral and cerebral thrombotic diseases. The benefit of using thrombin-specific inhibitors alone in acute myocardial infarction or unstable angina remains uncertain. Some of thrombin-specific inhibitors which are small molecules are orally active. The major concern for the use of thrombin-specific inhibitors is bleeding complication. The efficacy, safety, stability and oral bioavailability may be considerably improved through structural optimization. A growing line of evidence suggests that statins, the most commonly prescribed cholesterol lowering drug, may inhibit thrombin generation. Statins do not cause bleeding and have an outstanding safety profile. The findings suggest that further development of thrombin-specific inhibitors and exploration of the potential applications of non-specific thrombin inhibitors, including statins, may improve the prevention and management of thromboembotic events.

2. INTRODUCTION

Thromboembolization is one of the most common underlying causes of death and disability. Management of thromboembolic diseases, including myocardial infarction, stroke, deep vein thrombosis and pulmonary embolism, has become a socioeconomic burden in industrialized countries (1). Pharmacological prevention of thromboembolization with a good safety profile is highly demanded. Thrombin, the key enzyme and product of coagulation cascade, promotes coagulation at all levels. It is also implicated in the processes of cell proliferation, inflammation and tissue remodeling (2). Heparin is a classical anticoagulant for the treatment of thromboembolic disorders. It binds to antithrombin III and indirectly inactivates thrombin in fluid phase (3). Heparin is not effective for thrombin embedded in thrombus and caused some harmful effects. The most frequent side effect of heparin is hemorrhage. Major bleeding or cerebral hemorrhage during heparin are often catastrophic. Low molecular weight heparin (LMWH) moderately reduced hemorrhagic risk compared to unfractionated heparin but still caused significantly higher incidence of bleeding episodes compared to placebo in meta-analysis (4). An uncommon, but serious, adverse effect of heparin is haprin-induced thrombocytopenia (HIT) (5). HIT leads to extensive thromboembolism and hemorrhage with 25%-30% of mortality (1,. Coumadin (Warfarin), a vitamin K antagonist, is a commonly used oral anticoagulant but has a slow onset and wide interactions with other medications or diets (7). Thrombin-specific inhibitors have been considered as an effective and relatively safe alternative for the classical anticoagulants. A growing battery of compounds was considered as peptidomimetic thrombin-inhibitors (8, 9, 10). Some of them have been applied in clinical trials. Experimental and clinical results demonstrated that common cholesterol lowering drugs, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors or statins, attenuated the generation of thrombin (11). This review summarizes up-to-date information on the development and applications of thrombin-specific inhibitors and the potential effects of statins on thrombin inhibition.

3. LEECHES AND HIRUDIN

Leeches, *Hirudo medicinalis,* were used for bloodletting to treat medical disorders long before recorded history (12). A single chain polypeptide, named as hirudin, was isolated from slavery fluid of leeches in 1950s (13). Hirudin, the 65 amino acid polypeptide with an approximate molecular weight of 7 kDa, specifically binds and inhibits the activities of thrombin. It has been considered as the most potent natural thrombin-specific inhibitor. Hirudin irreversibly binds in high affinity with clot-bound or fluid phase thrombin at the active sites of thrombin in 1:1 stoichiometric manner (14). Hirudin does not inhibit platelet aggregation but prolongs activated partial thromboplastin time (aPTT) and bleeding time. Desirudin, a synthetic hirudin analogue, was clearly more effective for the prevention of thromboembolic events after orthopedic surgery than regular or LMWH and was associated with similar incidence of hemorrhagic events (15, 16). The outcomes of hirudin treatment from trials in acute myocardial infarction were controversial. Several large trials using hirudin in patients with coronary artery disease were suspended due to unexpectedly high incidence of severe bleeding complications (17-19). The incidences of major or fatal hemorrhage of low dosages of recombinant hirudins in patients with acute coronary syndromes were similar to that using heparin (20, 21). Intravascular transfer of hirudin gene reduced balloon catheter injury-induced neointima formation in rat carotid arteries (22). Results of Helvetica study indicated that intravenous or subcutaneous administration of hirudin reduced cardiac events < 24 h after angioplasty in patients with stable angina, but did not provide long-term benefits to the prevention of restenosis partially due to its side effects (23). Modifications of regimen may considerably improve the efficacy of hirudin in the prevention of restenosis in animal models (24). The application of hirudin and its analogues in the prevention of thrombosis was limited mainly due to hemorrhage. The major concern for the application of recombinant hirudins is high incidence of hemorrhage.

4. INHIBITION OF THROMBIN ACTIVE SITE

The crystallography of thrombin promotes the understanding of the structure and activity of thrombin, which also lead to rationale design of thrombin-specific inhibitors. The catalytic active site of thrombin is composed of a serine^{195}-histidine^{57}-asparate^{102} structure (7). The structure of the active site results in the cleavage of natural substrates of thrombin at the position of arginine. Thrombin inhibitors possess following moieties which mimic the structures of the substrates of thrombin to simultaneously occupy three pockets near the catalytically active site of thrombin: i) an arginine, arginine aldehyde or benzamidine (P1 residue) in S pocket; ii) a proline or small hydrophobic element in P pocket; iii) a D-Phe or analogous hydrophobic group in D pocket (Figure 1). Available thrombin-specific inhibitors, including hirudin, meet all or some of these structural requirements.

5. SYNTHETIC PEPTIDE THROMBIN INHIBITORS

John W. Fenton II and his collaborators combined the fragments of hirudin inhibiting catalytic site (25) and fibrinogen recognition exosite of thrombin (26) to generate a new type of anti-thrombin peptides, which were named as hirulogs. Hirulog-1, a 20 amino acid polypeptide with its N-terminal as D-Phe-Pro-Arg-Pro followed by four consecutive glycines and a tail fragment of hirudin homologous to an extracellular portion of the thrombin receptor, is one of most potent hirulogs (27). Hirulog-1 (bivalirudin, Hirulog^{TM} or Angiomax) inhibits both catalytic and binding activity of thrombin (Figure 2). Hirulog-1 is a reversible thrombin inhibitor and its anti-coagulation activity is weaker than hirudin, which is partially due to cleavage at its Arg-Pro bond in the presence of α- and ζ-thrombin (28). Hirulog-1 treatment reduced cardiac events in patients after acute myocardiac infarction (29) or angioplasty in adjunct to streptokinase (30).

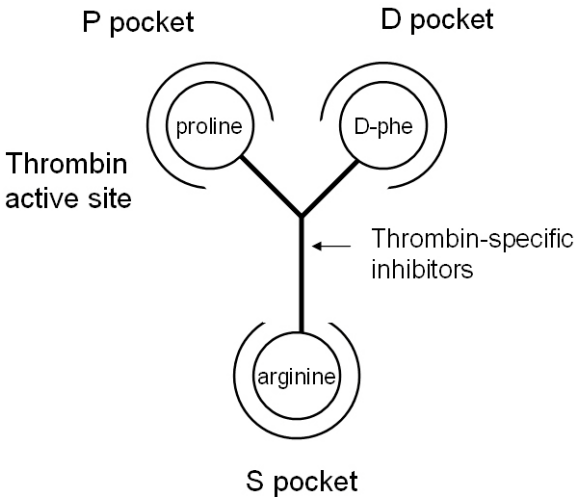

Figure 1 Schematic presentation of interactions between thrombin-specific inhibitors and the active site of thrombin.

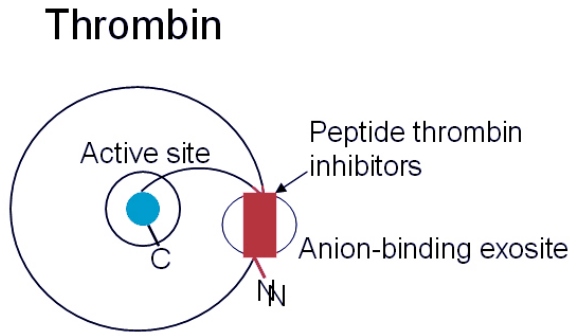

Figure 2. Scheme for the interactions between thrombin and peptide thrombin inhibitors.

Administration of hirulog-1 immediately before angioplasty reduced the incidence of procedure failure in comparison to heparin (31). Hirulog-1 was also used as an adjunctive therapy with thrombolytic agent in post-myocardial infarction (32) or with platelet inhibitors in post-stent implantation patients (33). Hirulog-1 attenuated platelet deposition on injured intima of rat carotid artery (34) and thrombin-induced generation of plasminogen activator inhibitor-1 (PAI-1, the major physiological inhibitor for fibrinolysis) from cultured arterial smooth muscle cells (35). Hirulog-1 reduced balloon catheter injury-induced neointima formation in rat carotid arteries (36) and restenosis in femoral arteries of atherosclerotic rabbits (37). The effect of hirulog-1 on injury induced vascular cell proliferation may be partially due to its inhibition on the expression of platelet-derived growth factor (PDGF) in neointima (36). Hirulog-1 caused less major bleeding than heparin but it still increased the incidence of haemorrhage in patients with unstable angina after angioplasty (31). Animal studies demonstrated that hirulog-1 infusion at effective doses significantly prolonged bleeding time and aPTT (36). Hemorrhage remains as a concern for treatment with hirulog-1 in humans, especially in clinically stable patients, when large dosages are required.

Several other peptide thrombin inhibitors were documented. dCha-Pro-N(Me)Arg-Thr-$(Gly)_5$-DYEPIPEEA-Cha-dGlu, or I-11, is a relatively stable thrombin inhibitor compared to hirulog-1 (38). CVS995, a 19 amino acid peptides, inhibited platelet aggregation and venous thrombosis in greater extent than hirulog-1 (39). Treatment with BCH-2763 inhibited thrombosis or injury-induced neointima formation in similar extent as hirulog-1 (40). Hirulog-like peptide (HLP) reduced neointima formation in balloon-injured rat carotid artery and restenosis in double-injury-induced atherosclerotic rabbits (41, 42). HLP treatment attenuated the expression of PDGF, tumor growth factor-beta and tissue factor in neointima of rat or rabbit carotid arteries. HLP treatment at effective dosages caured significantly less elongation of bleeding time or aPTT compared to hirulog-1 or heparin. HLP is composed of all natural amino acids, which allows it to be produced in large-scale through expression vector (41, 42). All these peptide thrombin inhibitors have to be administrated via parental routes due to rapid digestion in gastrointestinal tract or poor cell permeability.

6. SMALL MOLECULE THROMBIN INHIBITORS

The targets for the design of small molecular thrombin inhibitors include: i) effective inhibition of thrombin activity with minimal structural requirement; ii) increasing oral bioavailability of the drug; iii) no or less bleeding. Over 100 of small molecular thrombin inhibitors have been documented. The biochemical aspect of small molecular thrombin inhibitors has been extensively discussed in previous reviews (1,. The present review briefly describes on the classification and applications of small molecular thrombin inhibitors.

Small molecular thrombin inhibitors were derived from either tripeptide arginine aldehydes (D-Phe-Pro-Arg) or N-α-tosylarginine methyl ester (TAME). The efficacy and bioavailability of thrombin inhibitors was improved through modifying the structures of their moieties. The strategy of the modifications may be summarized into two principles: i) to maintain the basicity of the group (P1 residue) in S pocket but modify the moiety in P or D pocket; ii) to reduce high basicity in P1 residue. Resultant products may be further categorized into following four classes: tripeptidomimetic-transition state analogues (TSA), tripeptidomimetic, argatroban-type and N-α-arylsufonyl-4-amidinophenylalanine (NAPAP)-type analogues (10).

Argatroban is a derivative of TAME with an arginine side chain (43). Argatroban has been approved for several clinical indications to prevent thrombosis (44). Argatroban is able to inhibit non-thrombin serine proteases (45). The effect of argatroban on bleeding is similar as heparin (46). Argatroban has a low oral availability due to its guanidine moiety which reduces cell permeability. Efegatran is a derivative of tripeptidomimetric-TSA compounds (47). It was applied in clinical trials but has not been further developed. Inogatran is a tripeptidomimetric compound developed by Astra (48). It has a trypsin activity in the range of doses inhibiting thrombin activity (49). The

results of TRIM trials indicated that inogatran was less effective than heparin on ischemic events in patients with unstable coronary artery disease (50).

7. ORALLY ACTIVE THROMBIN INHIBITORS

Oral bioavailability of a drug may be affected by its stability in gastrointestinal tract, absorption, cell permeability or clearance. A compound with an oral bioavailability above 20% is considered to be acceptable for oral administration (51). Melagatran is a structural analogue of inogatran, but has high oral bioavailability. Administration of melagatran through nasal route reduced thrombus formation in canine coronary arteries with limited changes in aPTT (52). Melagatran is currently tested in phase II clinical trial. BMS-189664 is a compound converted to melagatran after oral administration and inhibited thrombosis in animal models (53). UK-156406 is an argatroban-type compound and displayed a half-life of 0.8 h and a high oral bioavailability in dogs (9). RGH-2958 (D-Phe-L-prolyl-L-Arg aldehyde sulfate) and DuP 714 (Ac-D-Phe-Pro-boro-Arg) were reported as effective thrombin inhibitors with high oral bioavailability (54, 55). BSF 208791, a novel orally available thrombin inhibitor, showed a better anti-thrombin properties associated with less elongation of bleeding time in rabbits compared to hirudin or LMWH (56). Oral administration of CI-1028 (PD172524 or LB30057), a benzamidrazone-derived thrombin inhibitor, effectively reduced venous thrombosis in canine model. Increased bleeding time was detected in animals using high doses of CI-1028 (57).

8. NON-PEPTIDE THROMBIN INHIBITORS

XU-817 has an arginine mimic structure, which displayed efficacy in preventing thrombus formation in rat vena cava thrombosis model after intravenous administration (58). L374087 is an orally available non-peptide thrombin inhibitor with a structure of pyridinone acetamide. Oral administration of L374087 reduced thrombus mass in coronary arteries and the sizes of myocardial infarction in dog model (59). Diarylsulfomanides (BM141248, 3DP-4147) and dibasic benzo[b]thiophene derivatives (LY 333545) has good anti-thrombin activity and high oral bioavailability in animals (9). Aptamers are oligonucleotides (DNA or RNA) that directly interacted with thrombin (60, 61). Some aptamers effectively reduced the formation of arterial thrombus in animal models (62).

9. CLINICAL APPLICATIONS OF THROMBIN INHIBITORS

The most widely accepted indication of the thrombin-specific inhibitors is HIT. Thrombin-specific inhibitors are currently considered as the best choice for a prompt anti-coagulation in HIT. The major rationale is that thrombin inhibitors are structurally unrelated to heparin. They are not affected by antibodies against heparin, which are the cause of HIT. Recombinant hirudins are approved for the treatment of HIT in the United States. Bivalirudin was reported to be successful in the treatment of patients with HIT (1). The ideal strategy for HIT treatment is to initiate the infusion of a thrombin-specific inhibitor as soon as the diagnosis is established. At the same time, an oral anticoagulant, usually warfarin, is started. After the effect of warfarin becomes stable, the thrombin inhibitor may be tapered and finally discontinued. Desirudin was successfully used in the prevention of venous thrombosis after hip or knee surgery (63). Argotroban has been approved for in the treatment of limb ischemia caused by obstructive arterial diseases or cerebral arterial thrombosis in Japan and for the treatment of HIT in USA (64, 45). The benefits of recombinant hirudins, bivalirudin or argatroban in patients with acute myocardial infarction and unstable remain unclear. No significant difference in the primary end-points (death or reinfarction) was detected between r-desirudin and heparin groups in GUSTO IIb (20) or TIMI 9B trial at 30 days after the occurrence of the ischemic events (21). The incidence of reinfarction or death within 24 h was significantly reduced in GUSTO IIb (65), but not in TIMI 9B trial (21). In patients with stable angina, desirudin caused lower incidence of death or myocardial infarction within 24 h compared to conventional heparin treatment (20, 64). Bivalirudin reduced the incidence of procedural failure in a subgroup of post-infarction patients in one trial but did not significantly affect the primary end point in both studies (66). Federal Drug Administration approved the use of bivalirudin in adjunct to aspirin following angioplasty in patients with unstable angina to prevent acute thrombosis. Most of small molecular thrombin inhibitors are currently investigated in pre-clinical stages. Some of them, including melagatran and UK-15406, were tested in early phases of clinical trials (10).

The anticoagulation effect of thrombin inhibitors is usually monitored using prothrombin time or aPTT. Ecarin clotting time was developed for monitoring the action of anti-thrombin agents. Ecarin is a component of snake venom (*Echris carinatus*), which converts prothrombin to meizothrombin which promotes clot formation in citrated whole blood or plasma samples (1).

The dosages for all thrombin-specific inhibitors in various indications have not been well standardized at this moment. The costs of anti-thrombin polypeptides are presently higher than heparin or LMWH for any indication. Cost-effectiveness may be considered as acceptable when thrombin-specific inhibitors are the only or the best choice for a particular clinical indication.

Thrombin inhibitors were often developed for specific indications. Effectiveness of one type of thrombin inhibitor for a specific indication may not necessarily be the same for other types of thrombin inhibitors. Thrombin inhibitors not only reduce the formation of fibrin but also affect many other biological activities. Various thrombin inhibitors may exert different effects on cellular activities.

10. STATINS ON THROMBIN GENERATION

Statins are rate-limiting enzyme for cholesterol synthesis. Several landmark clinical trials demonstrated that statins reduced cardiac death or events by 20-40% in

patients with coronary artery disease or diabetes. The cardiovascular benefits of statins were also seen in patients with normal cholesterol levels (67-71). Growing lines of evidence suggest that the cardiovascular beneficial effects of statins may partially result from their non-cholesterol lowering or pleiotropic effects on inflammation, coagulation, fibrinolysis, platelet activation, oxidative stress and cell proliferation (72). Earlier studies demonstrated that simvastatin administration reduced the expression of tissue factor in monocytes (73). The potential role of statins on thrombin inhibition and thrombosis prevention has been raised years ago (74). Recent studies in our group demonstrated that simvastatin treatment decreased the levels of pro-thrombin fragment 1+2 (F1+2), the marker of thrombin generation, and PAI-1 in type 2 diabetic patients. The levels of F1+2 directly reflect the generation of thrombin. The cholesterol levels in the patients positively correlated with PAI-1 but not F1+2, which suggest that statins may attenuate coagulation and improve fibrinolysis through cholesterol-dependent and non-cholesterol-dependent mechanisms (75). Statins inhibit the generation of a group of isoprenoids, which are involved in the modification of intracellular signaling proteins including small GTPases. Some of cellular activities of statins are expected to be derived from their effects on intracellar signaling (76, 77). Statins are orally administrated and do not cause bleeding. Large clinical trials demonstrated that statins have excellent safety profile. Myopathy was rarely found in patients receiving statins, which may largely prevented by careful monitoring and avoid drug cross-reactions (78). They are potentially to be used as preventive medications for populations with high risk of thrombosis. The preventive effects of statins on thromboembotic events require to be verified through large clinical trials.

11. SUMMARY

Thrombin-specific inhibitors have been developed based on the structure-function relationship between thrombin active sites and substrates. Desirudin, bivalirudin and argatraban were effective in the treatment of HIT or the prevention of thrombosis after orthopedic surgeries. The benefits of thrombin inhibitors in coronary artery disease and restenosis have not been conclusive. Most of small molecular thrombin inhibitors were tested in preclinical or early stages of clinical trials. The efficacy, safety, stability and oral bioavailability of small molecular thrombin inhibitors may be improved through structural modifications. Thrombin inhibition may be achieved by non-specific thrombin inhibitors, including statins.

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