Finding of Novel Anti-Thrombotic Drug via Selective Inhibition of Extrinsic Coagulation Pathway

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CHAPTER I: Introduction

Concept of specific inhibition of extrinsic coagulation cascade

Hemostasis, the physiological response to vascular injury, results in the formation of a hemostatic plug that prevents blood loss. Under normal conditions, factors that promote blood coagulation are balanced by those that inhibit it. Pathologic thrombosis occurs when procoagulant stimuli overwhelm natural anticoagulant and fibrinolytic systems. Such stimuli include vessel wall damage, inflammation, stasis and the excessive activation of coagulation. Damage to the vascular wall and the resultant exposure of tissue factor (TF)-expressing cells to blood initiate coagulation in both the arteries and veins [1]. Inflammatory cytokines generated after trauma, surgery, or medical illness can contribute to the procoagulant state. Endothelial cells are activated by inflammatory cytokines and express adhesion molecules that tether leukocytes to their surface. Bound monocytes produce TF and express the receptors for factor X and fibrinogen, and they promote coagulation [2, 3]. There are many reports that atherosclerotic plaques contain tissue factor, which is expressed by macrophages, smooth muscle cells and endothelial cells [4, 5]. TF was also seen in the lipid-rich core of atheromas, apparently associated with highly procoagulant microparticles derived from apoptotic cells, activated platelet and inflammatory cell [1]. Oxidized cholesterol and elevated glucose level can also induce TF expression and statins are known to lower TF levels by long-term treatment. This TF in pathogenic states is functionally active and competent to elicit thrombus formation when exposed to flowing blood. Venous thrombi develop under low-flow conditions and usually originate in the muscular veins of the calf or in the valve cusps pockets of the deep calf vein. Because the venous valve cusps are avascular, they depend on the circulating blood for their oxygen and nutrient supply.

Local hypoxemia occurs with venous stasis and can induce TF expression in the valve cusps. Also a high risk for venous thrombosis is considered to be partially due to TF-containing microparticle release during tissue damage. In addition to these etiology results for TF, epidemiological analysis shows that high plasma levels of factor VIIa (FVIIa) correlate with high morbidity of coronary heart disease (CHD) and myocardial infarction (MI) [6]. Some genome wide association studies indicate that FVIIa levels are changed by R353Q polymorphism [7] and that FVII ins-323 allele decreases the frequency of stroke in atrial fibrillation (AF) patients [8]. Also the CHD events lowering effect due to statins is known to be affected by FVII SNPs [9].

Since it is generally accepted that thrombus formation is initiated by TF *in vivo* [1], drugs that target the TF/VIIa complex should be potent anticoagulants. Theoretically, either inhibition of TF/VIIa complex formation or inhibition of its active-site which is responsible for cleavage of mainly factor X will disable the extrinsic coagulation pathway (Fig.1).

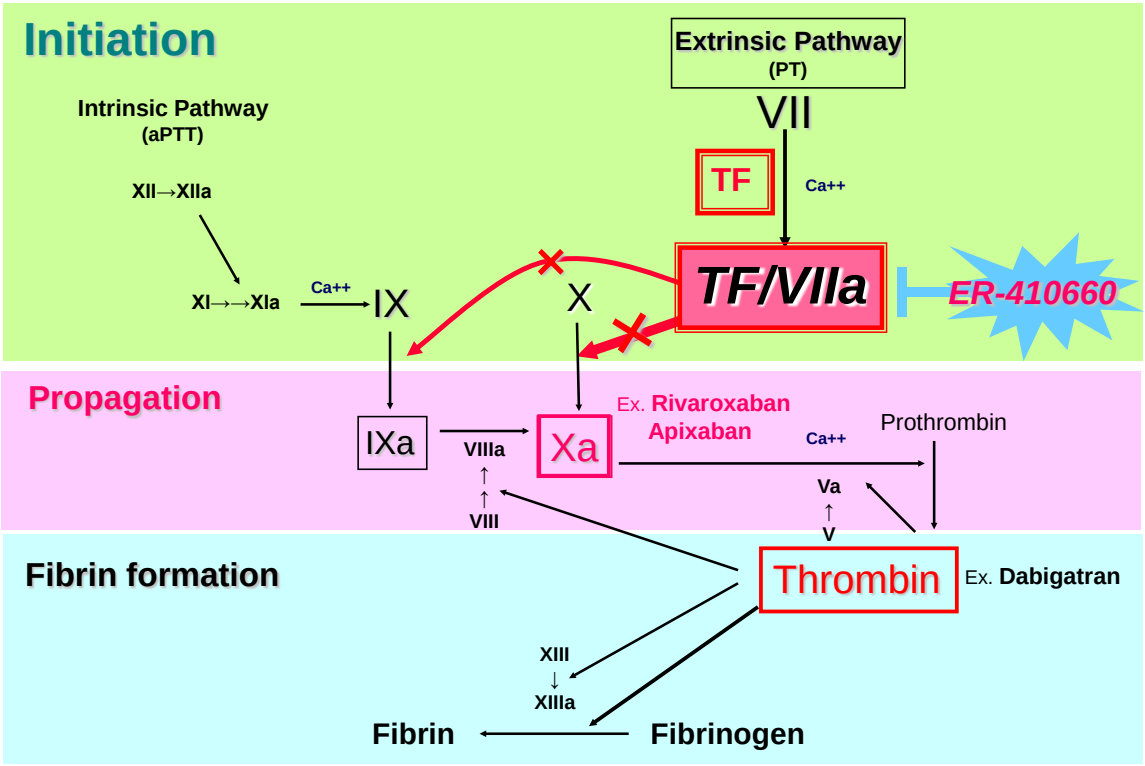


Fig.1. Blood coagulation cascade and mechanism of action for achieving extrinsic coagulation specific inhibition

The intensive search for novel anticoagulants reflects the significance and impact on the healthcare system of both thrombosis and its prevention. Antithrombotics are needed for short-term use in the setting of acute medical conditions, and also for the long-term prevention of thrombotic events. While for short-term use, parenteral administration of drugs is most desirable, oral administration is the goal for long-term medication. Considering also the major concern of anticoagulation, i.e. bleeding complications, an ideal antithrombotic drug should fulfill the following requirements.

- good intestinal absorption
- adequate plasma protein binding
- appropriate half-life with a duration giving good compromise between convenience and safety
- predictable pharmacokinetic and pharmacodynamic profiles with rapid onset
- no need for anticoagulation activity monitoring
- no food or drug interactions
- no risk of thrombocytopenia
- a reversible action

Many companies are focusing on the development of direct and selective coagulation inhibitors that attempt to fulfill the requirements mention above [1, 10, 11]. New anticoagulant strategies have targeted blocking the initiation step, preventing the propagation step, or inhibiting thrombin. The initiation step can be stopped by agents that affect the TF/VIIa complex, whereas the propagation step can be blocked by drugs that inhibit factor IXa or factor Xa. Finally, thrombin inhibitors prevent fibrin formation

and block thrombin-mediated feedback activation. There are many candidate drugs that target thrombin and factor Xa, for example dabigatran, rivaroxaban, apixaban, edoxaban, betrixaban and YM150 have progressed to clinical testing [12, 13]. The front-running oral agent is dabigatran [14, 15], rivaroxaban [16, 17, 18], and apixaban [19] which has entered the global markets for prevention of venous thromboembolism and cardioembolic stroke. It has become obvious that excessive inhibition of factors common to both coagulation pathways can lead to bleeding complications. On the other hand, TF/VIIa complex inhibitors may prove to be enhanced antithrombotic drugs with decreased bleeding risk, because they leave intact the intrinsic pathway, so maintaining normal hemostasis. Although TF/VIIa complex is a proper and attractive target, only a few parenteral drugs that target this complex have been evaluated in clinical trials [1, 4, 21-30]. These include anti-TF antibody [4, 21, 26], recombinant tissue factor pathway inhibitor (TFPI) [1, 4, 22], active-site blocked factor VIIa (FVIIai) [1, 4] and nematode anticoagulant peptide (rNAPc2) [27-29]. NAPc2 is isolated from the saliva of the hookworm *Ancylostoma caninum* and interferes with TF activity by binding to factor Xa or factor X to form an inhibitory complex with TF/VIIa. Several studies in primates, as well as first studies in human revealed very promising results in a phase II clinical trial, which were that rNAPc2 indeed appeared safe and effective in preventing overall postoperative venous thromboembolism in patients undergoing total knee replacement, and inhibited thrombin generation during coronary angioplasty. However, all the development programs of rNAPc2 including those for cancer were terminated by Nuvelo due to the company’s present financial problems. Furthermore, the development of a small molecule, direct TF/VIIa active-site inhibitor as an oral anticoagulant has yet to be achieved [30].

Epidemiology of antithrombotic drug market

The cardiovascular market is the biggest segment of the pharmaceutical market. In 2002, sales of cardiovascular products exceeded $50 billion and accounted for nearly 20 % of prescription pharmaceutical sales in the seven major markets (United States, France, Germany, Italy, Spain, United Kingdom and Japan). This market comprises five clearly defined segments, of which antithrombotics constitute the third-largest (about 13 %), and the only one that has grown at a rate similar to the antidyslipidemic segment. In the United States, the anticoagulant market has grown at 18 % per year and the antiplatelet market at 34 % per year. Antithrombotic agents are used for the treatment of a number of major cardiovasucular conditions, including acute ischemic stroke, acute myocardial infarction, atrial fibrillation, venous thrombosis/pulmonary embolism and unstable angina. Epidemiology of select cardiovascular conditions in the major markets is provided in the table below.

Tabale 1 Epidemiology of select cardiovascular conditions in the major markets

Source: Decision Resources, Inc

	2002 (000s)	%	2007 (000s)	%	2012 (000s)	%	2002-2012 Growth (%/year)
Acute ischemic stroke							
Events	1357.0		1478.6		1625.6		1.8
Diagnosed (% of total)	1154.7	85.1	1302.1	88.1	1464.3	90.1	2.4
Acute myocardial infarction							
Events	729.8		786.9		857.1		1.6
Diagnosed (% of total)	729.8	100	786.9	100	857.1	100	1.6
Atrial fibrillation							
Events	7412.0		7963.8		8616.5		1.5
Diagnosed (% of total)	5772.4	77.9	6488.3	81.5	7021.6	81.5	2
Venous thrombosis/pulmonaru embolism							
Events	1190.8		1280.8		1382.8		1.5
Diagnosed (% of total)	1190.7	100	1280.8	100	1382.8	100	1.5
Unstable angina/non-ST-elevation myocardial infarction							
Events	2762.9		2985.5		3250.1		1.6
Diagnosed (% of total)	2762.9	100	2985.5	100	3250.1	100	1.6

Atrial fibrillation is generally treated with oral antiplatelet agents, principally aspirin for low-risk patients and anticoagulants for patients at greater risk. Acute ischemic stroke is treated symptomatically with oral agents. Acute myocardial infarction is treated with a variety of antithrombotic agents. Oral antiplatelet agents are used both during treatment and subsequently for prophylaxis, while intravenous therapeutics of various classes are used in the acute setting. Patients with a significant risk of developing venous thrombosis are generally treated with anticoagulants, and usually for prolonged periods, with intravenous agents widely used after major surgery. Unstable angina is generally treated prophylactically with oral agents, the use of intravenous therapies being confined to the acute setting.

The $17 billion antithrombotics market has maintained consistent growth over the 2004-2008 period in seven major markets (US, Japan, France, Germany, Italy and UK). Among the antithrombotics market, the value sales of anticoagulants have grown as well as that of antiplatelets.

[Antithrombotics: Will Generics Temper the Strong Sales Trend? Spectrum: 2004]

Trend in medical care and treatment for target diseases

Outline of current treatment for thromboisis

Thrombosis can occur in either the arterial or venous circulation. With respect to the arteries, most heart attacks and many strokes are triggered by thrombosis secondary to disrupted atherosclerotic plaques. Atrial fibrillation is another cause of stroke. Patients with this common rhythm abnormality have a five-fold greater risk of having a stroke because of their propensity for left atrial thrombosis and subsequent cerebral embolism. Venous thromboembolism (VTE) includes deep vein thrombosis (DVT) and pulmonary

embolism and is the third most common vascular disorder after heart attack and stroke. In contrast to arterial thrombosis, venous thrombi usually arise in areas where the vein wall is grossly intact. Sluggish blood flow, endothelial cell activation, and hypercoagulability predispose patients to DVT.

Thrombi are composed of platelet aggregates, fibrin and trapped red cells. Because arterial thrombi form under high-shear conditions, platelets are abundant and fibrin is relatively sparse. In contrast, venous thrombi, which form under low-shear conditions, are rich in fibrin and trapped red cells and contain fewer platelets. These features have important implications for antithrombotic therapy. Targeting the components of both arterial and venous thrombi, antithrombotic drugs encompass antiplatelet agents, anticoagulants, and fibrinolytic drugs. Because of the preponderance of platelets in arterial thrombi, antiplatelet drugs are the mainstay of their prevention and treatment. However, anticoagulants are also effective in this setting, although they are not widely used. Anticoagulants are the mainstay in the prevention and treatment of VTE because fibrin predominates in venous thrombi. Antiplatelet drugs are less efficacious than anticoagulants in reducing the risk of VTE, which is consistent with the scarcity of platelets in venous thrombi.

Despite the widespread use of antithrombotic drugs, thrombotic diseases continue to be a major cause of death and disability. Therefore, there remains a need for more effective therapies to combat these disorders.

Unmet medical needs in treatment with warfarin

Stroke prevention in patients with atrial fibrillation and prevention of recurrence in patients with unprovoked VTE require long-term anticoagulant therapy. For the past 65

years, the only oral anticoagulants that have been available are the vitamin K antagonists, such as warfarin. Although effective, warfarin has many limitations as follows.

Limitations	**Consequences**
Slow onset of action	**Necessitates overlap with rapidly acting parenteral anticoagulants**
Genetic polymorphisms affect metabolism	**Variable dose requirements**
Multiple food and drug interactions	**Coagulation monitoring required**
Narrow therapeutic window	**Coagulation monitoring required**

Because of these inconveniences, many patients with atrial fibrillation refuse to take anticoagulant therapy, thereby forgoing an opportunity for stroke prevention. Even when patients do take warfarin, therapeutic levels of anticoagulation are achieved only half the time. These problems highlight the need for more convenient oral anticoagulant drugs that can be given in fixed doses and will produce a predictable anticoagulant response such that little or no monitoring is required.

An overarching problem with antithrombotic therapy is the difficulty of striking the optimal balance between efficacy and safety, particularly bleeding. There is mounting evidence that bleeding is associated with adverse cardiovascular outcomes and carries the same risk of mortality that recurrent ischemia does. The adverse impact of bleeding has prompted an evaluation of antithrombotic regimens that maximize efficacy without increasing the risk of bleeding and highlighted the need for antithrombotic drugs that

attenuate thrombosis with minimal effects on hemostasis. Only by identifying new drug targets, these closely linked biological processes are likely to be separated.

Novel Anticoagulants

The greatest unmet medical need in anticoagulant therapy is a simple and convenient replacement for warfarin to streamline long-term treatment. Most of the attention has focused on the development of oral agents that target thrombin or factor Xa. However, the utility of a long-acting synthetic parenteral pentasaccharide, which also targets factor Xa, is under investigation.

Thrombin inhibitors

As the final effector in coagulation, thrombin converts fibrinogen to fibrin. Thrombin also amplifies its own generation by feedback activation of factors V and VIII, respectively. In addition, thrombin coordinates the process of platelet activation and aggregation with coagulation. Because of its multiple roles in coagulation, the inhibition of thrombin not only blocks fibrin formation but also attenuates thrombin generation and platelet activation.

The suitability of thrombin as a target was validated by clinical experience with ximelagatran, the first oral, direct thrombin inhibitor. Ximelagatran was shown to be not inferior to conventional anticoagulants for prevention and treatment of VTE and for stroke prevention in atrial fibrillation. Ximelagatran was withdrawn because of potential hepatotoxicity, but the favorable results with this drug confirmed long-term thrombin inhibition as an effective and safe strategy, at least with regard to the hemorrhage problem.

Factor Xa inhibitors

When factor Xa is assembled along with factor Va on the surface of activated platelets, the resultant prothrombinase complex is a potent activator of prothrombin. Consequently, inhibitors of factor Xa block thrombin generation. The orally administered factor Xa inhibitors are small molecules that bind to the active site of factor Xa in a reversible fashion. As a class, these drugs have high oral bioavailability, a rapid onset of action, half-lives that range from 7 to 15 h, and both renal and extrarenal mechanisms of excretion. Although direct comparisons of orally active inhibitors of factor Xa and of thrombin in animal models have suggested that, for similar antithrombotic efficacy, upstream inhibition at the level of factor Xa causes less bleeding than downstream blockade of thrombin, head-to-head trials in humans have not been performed. Nonetheless, in phase II trials, dose-dependent increases in the rates of bleeding are observed with inhibitors of either factor Xa or thrombin. Therefore, at this point, there is no evidence that one target is any better than the other.

Consequence

As the potential advantages of the new anticoagulants over warfarin, the new agents have been designed to be administered in fixed doses without the need for routine coagulation monitoring. This is possible because these agents are not affected by any food constituents and the potential for drug-drug interactions is low. It is forecasted that value sales of anticoagulants market will continue to grow in the next decade. Currently launched products and pipeline compounds are expected to lead the anticoagulants market. Rivaroxaban and dabigatran replacing enoxaparin are seen as the next

blockbusters in the anticoagulants market. In the search result from the published database, PCI-27483 (Pharmacyclics) alone is once being clinically developed (Ph I) as a small compound TF/VIIa inhibitor by subcutaneous administration. But, the development of PCI-27483 was frozen at this moment, though the reason is not obvious. So TF/VIIa inhibitors are thought to be an unexplored field of anticoagulants at this time. In the future, new products with varied MOA related to coagulation factors are expected to be marketed. TF/VIIa inhibitors will be evaluated in comparison with them in addition to warfarin from the viewpoints of efficacy, safety and convenience in the prevention or treatment of thrombosis.

[Keiser J, Vascular Pharmacology: Opportunities for Intervention. Clin Pharmacol Ther, 2009;86,139-46]

Target indications and populations

Venous thrombosis and pulmonary embolism

Deep vein thrombosis and pulmonary embolism (DVT/PE) represent a major public health problem. A 25-year population-based study published in 1998 found that the overall age- and sex-adjusted annual incidence of VTE was 1.17 per 1,000 (0.48 per 1,000 for DVT and 0.69 per 1,000 for PE). Applying these figures to today's population of approximately 300 million Americans suggests that more than 350,000 individuals are affected by DVT/PE each year [31]. But there is reason to believe that the true incidence rate could be significantly higher. Several studies suggest that these diseases are often undiagnosed. Some study of nursing home patients found that the condition was correctly diagnosed before death in only 39 to 50 % of patients where it was confirmed in an autopsy. Therefore as many as 700,000 cases of DVT/PE may occur in US each year. A 20-year review of data found that the age-adjusted death rate for PE

was 94 per 1,000,000 individuals. Extrapolating to today's US population suggests that an estimated 28,200 people die in each year. But as noted previously, PE is often undiagnosed, and thus the true death rate is almost certainly substantially higher. It is estimated that 30 % of patients will die within 3 months. Applying this 30 % figure to the previously estimation of 700,000 cases each year suggest that perhaps over 200,000 individuals die directly or indirectly as a results of DVT/PE in US. The clinical impact of DVT/PE is also highlighted by the fact that 10 % of hospital death can be attributed to PE, nevertheless it is the most common preventable cause of death. The morbidity and mortality of DVT/PE is not only confined to the first symptomatic acute event. A long-term complication of DVT is the post-thrombotic syndrome, consisting of chronic pain, swelling (or edema) and occasionally ulceration of the leg, which are observed in about 30 % of patients after 2 years. An immediate and adequate course of anticoagulant drugs is the mainstay therapy of DVT/PE in the acute phase as well as in post-acute phase for prolonged prevention of recurrent events.

The most common risk factors are recent surgery or hospitalization. And 40 % of these patients did not receive heparin prophylaxis. The chance of developing DVT/PE during or after a surgical procedure varies with the nature of the procedure, including its duration, and with perioperative care. A comprehensive body of research has established that pharmacologic measures to prevent DVT/PE in appropriate surgical patients are both effective and associated with a low risk of postoperative bleeding complications. Therefore, the risk/benefit ratio favors the prophylactic approach to treatment. In addition, this approach has proven to be cost-effective in moderate- and high-risk general surgery patients. Other risk factors include advanced age, obesity, infection, immobilization, use of hormonal contraception, smoking and air travel are some of the

better-known causes.

Major orthopaedic surgery, involving hip or knee replacement surgery or hip fracture surgery, is associated with a high risk of DVT/PE [32]. The risk results from stasis of venous blood flow as well as direct injury to the veins during surgery. Without prophylaxis, rates of DVT range from 40 % to 60 % when assessed by venography at 7 to 14 days after major orthopaedic surgery [33]. Routine ventilation-perfusion scans in patients following hip or knee arthroplasty revealed pulmonary emboli in 3 % to 28 % of patients. Initial anticoagulation for DVT/PE prophylaxis and treatment is either unfractionated heparin by continuous intravenous infusion, low-molecular-weight heparin (LMWH) by subcutaneous injection (once or twice daily), or fondaparinux by once-daily injections followed by treatment with an oral anticoagulant. Major risks associated with heparin are bleeding complications and heparin-induced thrombocytopenia (HIT) which is lower with LMWHs and fondaparinux. Patients with a history of HIT should receive an alternative anticoagulant, such as argatroban, lepirudin, or danaparoid. Osteoporosis is another potential adverse effect when heparin is administered for longer than a month. Long-term treatment generally requires a transition from heparin or fondaparinux to a vitamin K antagonist (VKA), such as warfarin. VKAs have a delayed onset of anticoagulant effect; following initiation of VKA therapy, heparin can be discontinued once the INR has reached the therapeutic range (2.0 to 3.0). Treatment with warfarin for four weeks to twelve months has been shown to reduce the risk of DVT recurrence by 90 %. One exception to this general approach is the cancer patient with DVT, in whom long-term treatment with warfarin is less effective in preventing recurrence [34]. LMWH, shown in one study to be roughly twice as effective in preventing this outcome, should be considered in these patients.

Cardio embolic stroke prevention in atrial fibrillation

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia, affecting approximately 2.2 million Americans [35]. The prevalence of AF increases with age, with 70 % of case occurring in patients between the ages of 65 years and 85 years. AF is also more common in men than in women at all ages. The prevalence and incidence of AF are expected to increase in the coming decades because of the aging of the population; improved survival rates associated with coronary heart disease, heart failure and hypertension; and increased rate of performance of surgical procedures. It is projected that 7.5 million individuals will have AF in the US by the year 2020 [36]. The economic burden of AF is substantial because of high rates of hospitalization and other health resource utilization. Ischemic stroke is the most devastating complication of AF. Risk factors for stroke in patients with AF include recent congestive heart failure, hypertension, advanced age, diabetes and history of stroke or transient ischemic attack. Risk scoring systems, such as CHADS2, have been developed to predict an individual's risk for developing AF and risk for stroke in AF patients. Oral VKAs like warfarin are extremely effective in reducing the risk of stroke among patients with AF. The early trial consistently demonstrated benefit with an overall 68 % risk reduction in stroke. Despite this dramatic efficacy, warfarin remains underused in clinical practice. Intracranial hemorrhage is the most feared complication of anticoagulant therapy. Mortality related to intacerebral hemorrhage approximates 50 % and is related to hematoma volume and hematoma expansion. Risk factors of intracranial hemorrhage include anticoagulation intensity, age, prior stroke, hypertension, concomitant antiplatelet therapy, and arterial vasculopathy. The paradox facing clinicians and patients is that many of the risk factors

for intracranial hemorrhage are also risk factors for ischemic stroke. When assessing risk versus benefit, current evidence from randomized controlled trail and observational studies weighs in favor of anticoagulant therapy. The rate of intracranial hemorrhage is approximately 0.5 - 0.6 %. The rate of major extracranial hemorrhage is 2 %. On the other hand, the rate of stroke among individuals with AF and multiple risk factors exceeds 10 %. However, there are important caveats to the published rates of major hemorrhage. Recent trials and most observational cohorts have largely reported the outcomes of prevalent users of warfarin. Because most major bleeding occurs early in the course of coagulation, enrollment of predominantly long-time users of warfarin will result in lower estimates of hemorrhage. This survival bias was illustrated by an inception cohort study of patients with AF aged 65 years and greater those were newly starting warfarin therapy. The rate of major hemorrhage in the first year was 7 % and discontinuation of warfarin was highest in the first 90 days. The cessation of warfarin was disproportionately high among patients at the highest risk of stroke.

Coronary artery disease/ myocardial infarction

Coronary artery disease (CAD) or atherosclerotic heart disease is the end result of the accumulation of atheromatous plaques. It is sometimes also called coronary heart disease (CHD), but although CAD is the most common cause of CHD, it is not the only cause. CAD is the leading cause of death worldwide [37]. While the symptoms and signs of coronary artery disease are noted in the advanced state of disease, most individuals with coronary artery disease show no evidence of disease for decades as the disease progresses before the first onset of symptoms, often a "sudden" heart attack, finally arises. Myocardial infarction (MI) or acute myocardial infarction (AMI),

commonly known as a heart attack, is the interruption of blood supply to part of the heart, causing some heart cells to die. This is most commonly due to occlusion of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids (fatty acids) and white blood cells (especially macrophages) in the wall of an artery. The resulting ischemia and oxygen shortage, if left untreated for a sufficient period of time, can cause damage or infarction of heart muscle tissue. Myocardial infarction is a common presentation of ischemic heart disease. The WHO estimated that in 2002, 12.6 % of deaths worldwide were from ischemic heart disease. Ischemic heart disease is the leading cause of death in developed countries, but third to AIDS and lower respiratory infections in developing countries. In the United States, diseases of the heart are the leading cause of death, causing a higher mortality than cancer [37]. Coronary heart disease is responsible for 1 in 5 deaths in the U.S. This means that roughly every 65 seconds, an American dies of a coronary event. In India, cardiovascular disease (CVD) is the leading cause of death. The deaths due to CVD in India were 32 % of all deaths in 2007 and are expected to rise from 1.17 million in 1990 and 1.59 million in 2000 to 2.03 million in 2010.

The patient will receive a number of diagnostic tests, such as an electrocardiogram (ECG). On the basis of the ECG, a distinction is made between ST elevation MI (STEMI) or non-ST elevation MI (non-STEMI). Most cases of STEMI are treated with thrombolysis or if possible with percutaneous coronary intervention (PCI, angioplasty and stent insertion), provided the hospital has facilities for coronary angiography. NSTEMI is managed with medication, although PCI is often performed during hospital admission. In patients who have multiple blockages and who are relatively stable, or in a few extraordinary emergency cases, bypass surgery of the blocked coronary artery is

an option.

The risk of a recurrent myocardial infarction decreases with strict blood pressure management and lifestyle changes, chiefly smoking cessation, regular exercise, a sensible diet for patients with heart disease, and limitation of alcohol intake. Patients are usually commenced on several long-term medications post-MI, with the aim of preventing secondary cardiovascular events such as further myocardial infarctions, congestive heart failure or cerebrovascular accident (CVA). Unless contraindicated, such medications may include antiplatelet drug, beta blocker, ACE inhibitor, statins and omega-3 fatty acids. Antiplatelet drug therapy, such as aspirin and/or clopidogrel, should be continued to reduce the risk of plaque rupture and recurrent myocardial infarction. Aspirin is first-line, owing to its low cost and comparable efficacy, with clopidogrel reserved for patients intolerant of aspirin. The combination of clopidogrel and aspirin may further reduce risk of cardiovascular events, however the risk of hemorrhage is increased [38].

Sepsis

Sepsis is a serious medical condition that is characterized by a whole-body inflammatory state which is called a systemic inflammatory response syndrome (SIRS) and the presence of a known or suspected infection. The clinical syndrome of sepsis is characterized by the disruption in homeostasis through uncontrolled inflammation, which response to microbes in the blood, urine, lungs, skin, or other tissues, coagulation and fibrinolysis [39-41]. In the US, sepsis causes an estimated 240,000 deaths/year and has related costs of US $20 billion/year. It has an annual increase of 1.5 – 8 %/year, probably as the result of increased number of patients with advanced age, compromised

immune status, increased use of invasive procedures and an increased prevalence of antibiotic-resistant organisms [34]. Despite enormous progress in intensive care therapy, the mortality rate of 30 – 50 % among septic patients has remained almost unchanged over the last 30 years. In Europe, the reported incidence of severe sepsis varies from 54 cases/100,000 peoples in Netherlands to 66 cases/100,000 people in the UK. The number of patients with severe sepsis treated in the UK has increased from 46/100,000 in 1996 to 66/100,000 in 2003. The mortality from severe sepsis in the EU may be 30/100,000/year corresponding to 110,600 deaths/year.

The therapy of sepsis rests on antibiotics, surgical drainage of infected fluid collections, fluid replacement and appropriate support for organ dysfunction. This may include hemodialysis in kidney failure, mechanical ventilation in pulmonary dysfunction, transfusion of blood products, and drug and fluid therapy for circulatory failure. Ensuring adequate nutrition, preferably by enteral feeding, but if necessary by parenteral nutrition, is important during prolonged illness. A problem in the adequate management of septic patients has been the delay in administering therapy after sepsis has been recognized. Published studies have demonstrated that for every hour delay in the administration of appropriate antibiotic therapy there is an associated 7 % rise in mortality. Most therapies aimed at the inflammation process itself have failed to improve outcome, however drotrecogin alfa (activated protein C) has been shown to decrease mortality from about 31 % to about 25 % in severe sepsis [42]. To qualify for drotrecogin alfa, a patient must have severe sepsis or septic shock with an APACHE II score of 25 or greater and a low risk of bleeding.

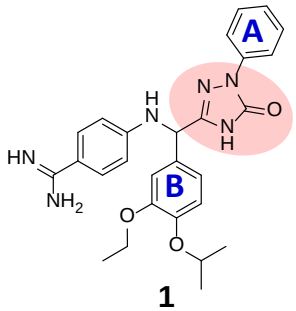
CHAPTER II: Discovery of ER-410660 and its prodrug form E5539

Compound optimization and finding of ER-410660 as a selective TF/VIIa inhibitor

Our drug discovery project was initiated to find a potent and selective TF/VIIa complex inhibitor. In September 1999, a high-throughput screening (HTS) system was established for finding inhibitors of the TF/VIIa complex formation or compounds blocking the FVIIa active-site. The HTS campaign was completed in December 1999, and resulted in the discovery of one Eisai original “trigger” compound. Synthetic optimization was next performed, but the hoped for improvement in TF/VIIa inhibitory potency could not be achieved. Therefore, Roche patent compound was selected instead as the starting point for product creation. As a result, we found good “seed” compounds which contains both an amidine and a carboxylic acid moiety. Choosing this approach left three issues to be solved. These were 1) oral bioavailability, 2) compound potency, and 3) patentability. The oral bioavailability of “seed” compound was very low because of poor membrane permeability. One of the reasons for this may be the strong basicity of the amidine group. However an amidine or equivalent moiety was indispensable for TF/VIIa inhibition. In fact, we did try to find an alternative to this amidine, but it was concluded that, at least for this template, it was impossible to replace the amidine effectively with another less basic moiety. Hence, at this point it was decided to opt for a prodrug strategy for solving issue 1).

Conversion of the carboxylic acid was effective for solving issues 2) and 3). Conversion into hydrazide derivatives maintains activity. However, active carboxylic acid derivatives, including these hydrazide analogues, were easily hydrolyzed back to the carboxylic acid in plasma. Next we tried converting the hydrazide linker moiety into a cyclic linker which was assumed to be harder to metabolism. Triazolone analogues

(compound 1) were prepared as these maintain the essential structural features of active amide and hydrazide analogues, a carbonyl group, an acidic proton and the possibility of aryl group attachment, while being resistant to hydrolysis (Fig. 2). Typical synthesis route of triazolone derivatives were shown in Fig.3.



1

FVIIa IC50 (μM)	1.1x PT[1] (μM)	2x APTT[2]/ 1.1x PT	Protease Selectivity Ratio		
			Xa	IIa	Trypsin
0.0026	12	11	5232	2335	749

Fig.2. Remarkable inhibitory activity of triazolone derivative on TF/VIIa activity and selectivity against other serine proteases. 1) The 1.1x PT is defined as the concentration of compound needed to prolong the clotting formation time by 1.1-fold in the prothrombin time (PT) assay. 2) The 2x APTT is defined as the concentration of compound needed to prolong the clotting formation time by 2-fold in the activated partial prothrombin time (aPTT) assay.

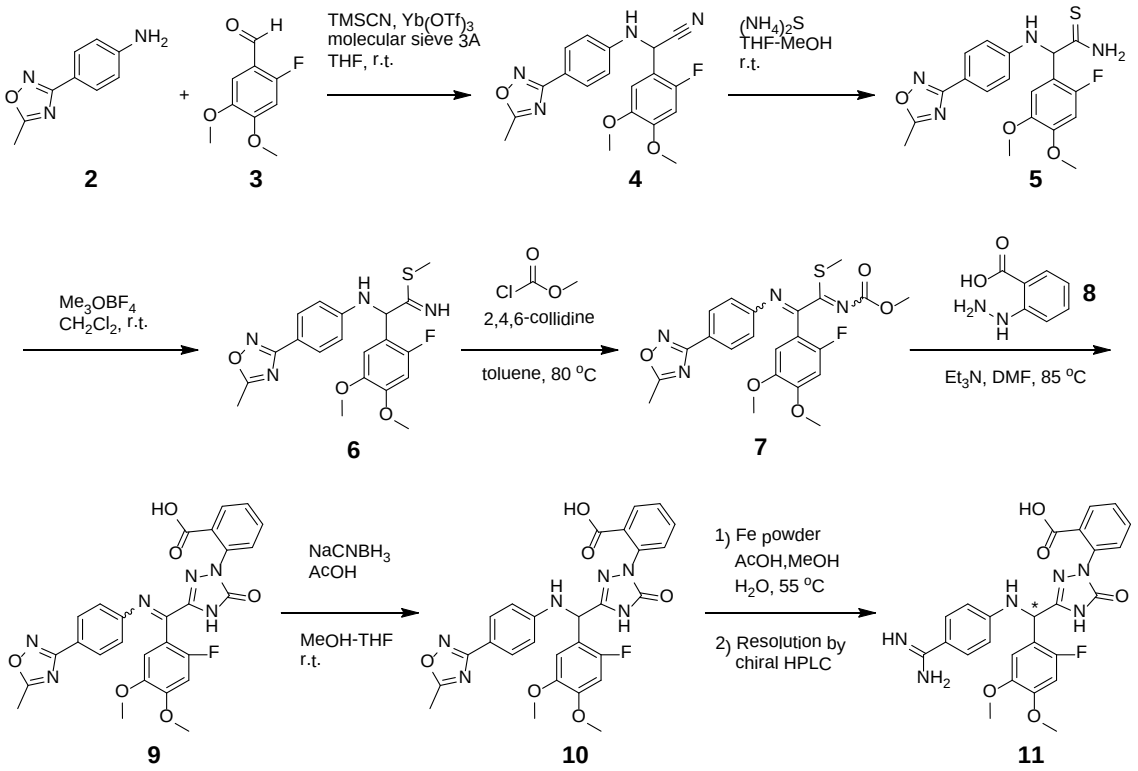
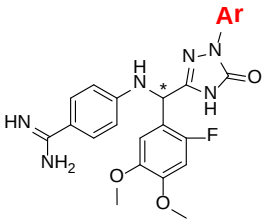
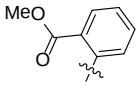
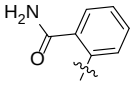
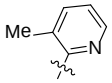
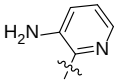
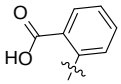
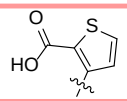
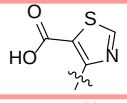
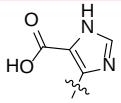


Fig.3. Typical synthetic route of triazolone derivatives as potent and selective TF/VIIa inhibitor.

Optimization of the B- and D-rings of triazolone analogues (compound 1) were started to find clinical developable TF/FVIIa inhibitors. Table 1 shows the SAR of A-ring optimization. When compared with lead compound 1 having an unsubstituted phenyl A-ring compounds having substituted phenyl or heteroaryl on the triazolone ring showed significant improvements in their selective inhibitory effects on extrinsic blood coagulation as indicated by PT assay results. Regarding substitution on the ring, ortho-substitution is well tolerated. Especially, a carboxy group shows potent inhibitory effects on the extrinsic pathway. The compounds 20, 21 and 22 having a carboxylic acid on a 5-membered heteroaryl display good selectivity against FXIa in comparison with benzoic acid 11.

Table 1: SAR of A-ring of triazolone derivatives

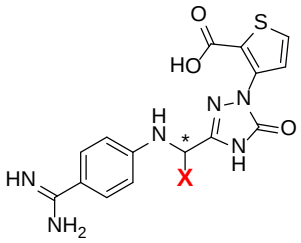


Cmpd #	Ar	FVIIa IC50 (μM)	1.1x PT[1] (μM)	2x APTT[2]/ 1.1x PT	Protease Selectivity Ratio Xa	IIa	Trypsin	XIa
12		0.0080	0.93	128	ND	1357	4	-
13		0.0079	0.68	118	ND	4975	141	-
14		0.0050	0.88	106	ND	6994	305	-
15		0.0067	0.37	200	ND	3910	63	-
16		0.0055	0.72	121	ND	281	372	-
17		0.0070	0.49	41	ND	398	206	-
18		0.0110	0.69	116	ND	513	342	-
19		0.0113	0.60	138	ND	1195	557	-
11		0.0025	0.21	12	ND	898	184	12
20		0.0033	0.39	572	ND	42849	522	477
21		0.0165	0.35	743	ND	ND	361	68
22		0.0090	0.35	537	ND	22591	1838	132

*All compounds are supplied as chial. 1) The concentration to prolong the PT by 1.1-fold. 2) The concentration doubling aPTT.

Since compounds 19, 20 and 21 had improved the potency and selectivity of the inhibitory effects on extrinsic blood coagulation, 2-carboxythiophene, 5-carboxythiazole and pyrimidine ring were selected as A-ring candidates for further optimization focusing on their combination with the B-ring (Table 2, 3 and 4). As for the SAR of the B-ring, a wide range of substituents on the phenyl is well tolerated (e.g., halogen, alkyl, alkene, alkyne, alkoxy, hydroxyalkyl and oxygen containing fused ring etc.); also, the phenyl can be replaced by pyridyl without adversely affecting activity.

Table 2: SAR of thiophene containing triazolone derivatives



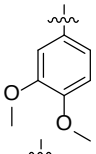
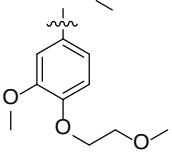
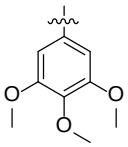
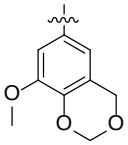
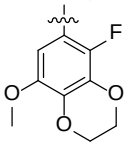
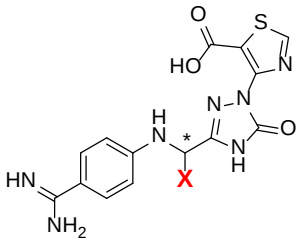
Cmpd #	X	FVIIa IC50 (μM)	1.1x PT (μM)	2x APTT/ 1.1x PT
23		**0.0023**	0.65	486
24		**0.0062**	0.53	185
25		**0.0045**	0.74	532
26		**0.0032**	0.80	519
27		**0.0028**	0.30	347

Table 3: SAR of thiazole containing triazolone derivatives



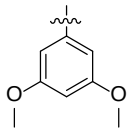
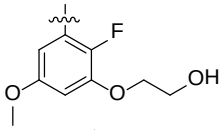
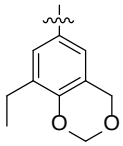
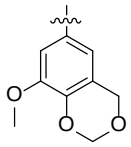
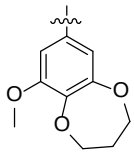
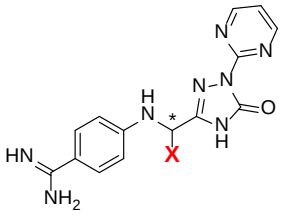
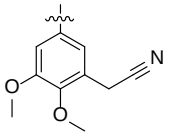
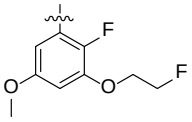
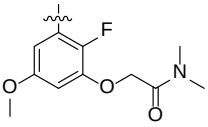
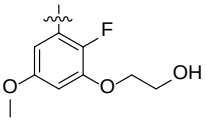
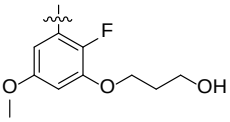
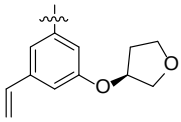
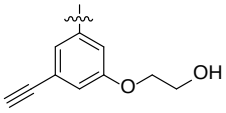
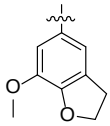
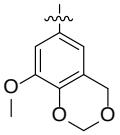
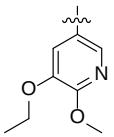
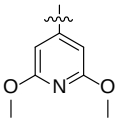
Cmpd #	X	FVIIa IC50 (μM)	1.1x PT (μM)	2x APTT/ 1.1x PT
28		**0.0237**	0.77	329
29		**0.0111**	0.39	190
30		**0.0099**	0.65	223
31		**0.0187**	0.42	826
32		**0.0101**	0.33	267

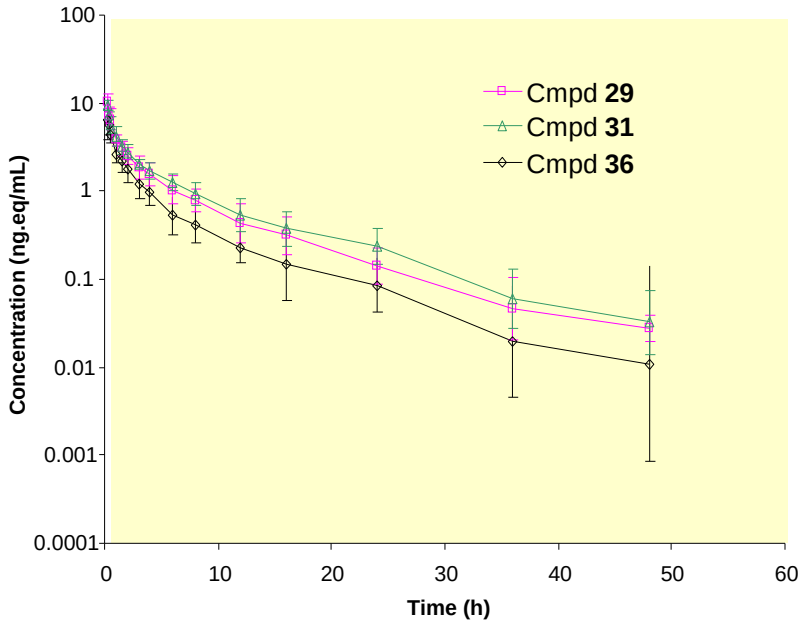
Table 4: SAR of pyrimidine containing triazolone derivatives



Cmpd #	X	FVIIa IC50 (μM)	1.1x PT (μM)	2x APTT/ 1.1x PT
33		0.0054	0.25	104
34		0.0048	0.26	142
35		0.0134	0.28	132
36		0.0048	0.33	194
37		0.0051	0.17	171
38		0.0013	0.39	110
39		0.0057	0.39	413
40		0.0071	0.30	183
41		0.0106	0.26	450
42		0.0094	0.19	621
43		0.0187	0.65	177

It should be noted that our drug target is in plasma, so during optimization, careful attention to plasma protein binding (PB) need to be paied. PB directly affects the activity of these compounds in plasma. To enhance their potency, consideration of not only VIIa inhibition but also PB (or hydrophilicity) was essential. In general, more polar compounds tend to have lower PB, and this is reflected in the fact that our compounds are very hydrophilic.

In vivo efficacy and bleeding time prolongation of a number of these compounds was evaluated in a rat model (detailed data not shown). The results clearly suggest that TF/VIIa plays a significant role in clotting formation and that it is a suitable target for development of a novel anticoagulant with low bleeding risk. As far as pharmacological potency is concerned, this series of triazolone derivatives has reached the level necessary for a promising candidate novel anticoagulant. Especially, compounds 29, 31 and 36 (ER-410660) were nominated for further prodrugging research to improve oral availability. However, it was difficult to predict the human pharmacokinetics (PK) profile of these inhibitors due to the poor reliability of prediction from animal scale-up. In addition, a human PK profile, especially half-life in plasma (t1/2), is important in the selection of the most suitable compound for its prodrugging research. Therefore, a human micro dosing study of compounds 29, 31 and 36 was conducted in Netherland. Summarized human PK results are shown in Fig. 4.



Cmpd #	t1/2 (h)	C_{max} (ng.eq/mL)	CL (L/h)	V_{ss} (L)
29	**9.4**	10.358	4.1	32.0
31	**8.7**	9.338	3.6	32.1
36	**9.2**	6.775	6.4	50.4

Non-compartment analysis

Study design
- ✓ Single dose, open-label, 3 period fixed sequence crossover
- ✓ 5 subjects, healthy male adult volunteers, to receive all 3 compounds as a microdose with 1 week washout between treatments
- ✓ Dose: 100 µg and 100 nCi [1)], given iv over 15 min infusion
- ✓ PK sampling for 48 h (plasma, urine and feces)

1) Labeled material for the study was prepared by Selcia Co. Ltd.

Fig.4. Results of human microdosing studies of compounds 29, 31 and 36 (ER-410660).

The t1/2 of each of the three compounds is about 9 hr and the human pharmacokinetics (PK) of the three compounds is very similar. They were not metabolized and were mainly excreted to urine. Thus all three compounds satisfy our PK criteria, and from this point of view anyone of them is suitable for further development. To address issue of oral availability, prodrug formation was attempted for compounds 29, 31 and 36 (ER-410660). From this initial examination, I concluded that ER-410660 had the highest potential for improving bioavailability by a prodrug strategy.

To understand the reason for bringing high potency against TF/VIIa complex and selectivity among various coagulation factors by ER-410660, the X-ray crystal structure of compound with FVIIa enzyme was analyzed. Fig. 5 shows the feature of specific interaction of ER-410660 to FVIIa enzyme.

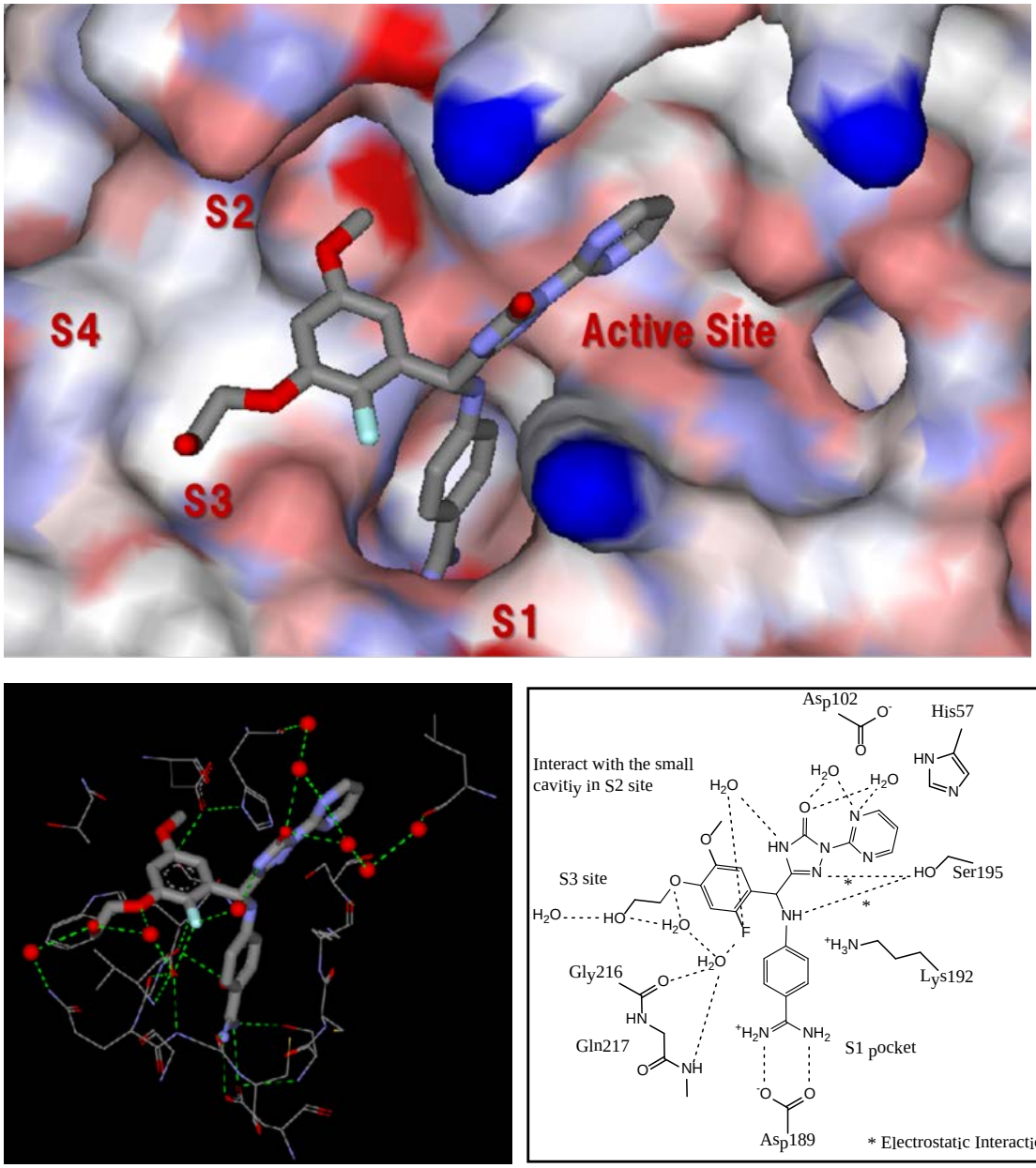


Fig. 5. X-ray co-crystal structure of ER-410660 with human FVIIa.

Discovery of E5539 as a prodrug form of ER-410660

As mentioned in CHAPTER II, three promising compounds were in hand, the potent and highly selective TF/VIIa inhibitors, such as compounds 29, 31 and 36 (ER-410660). Nevertheless, our active compounds had some issues that needed to be solved. These were 1) difficulty of human PK prediction 2) low bioavailability. It was needed to select one active compound for further prodrug investigation based on data on human PK, and the degree of BA improvement obtained in preliminary explorations. In order to solve issue 1), a human micro dosing study was conducted and human PK profiles were cleared. To address issue 2), prodrug formation was attempted for these compounds. One of the major reasons for the poor membrane permeability of ER-410660 may be their strong basicity, which is due to their amidine moiety. However, our initial studies showed that prodrug formation is necessary not only to mask the amidine moiety but also the triazolone moiety of compound. That is, it is necessary to mask both the amidine moiety with benzoate or carbamate and the triazolone as pivaloyl oxymethyl derivatives or esters (Fig. 6).

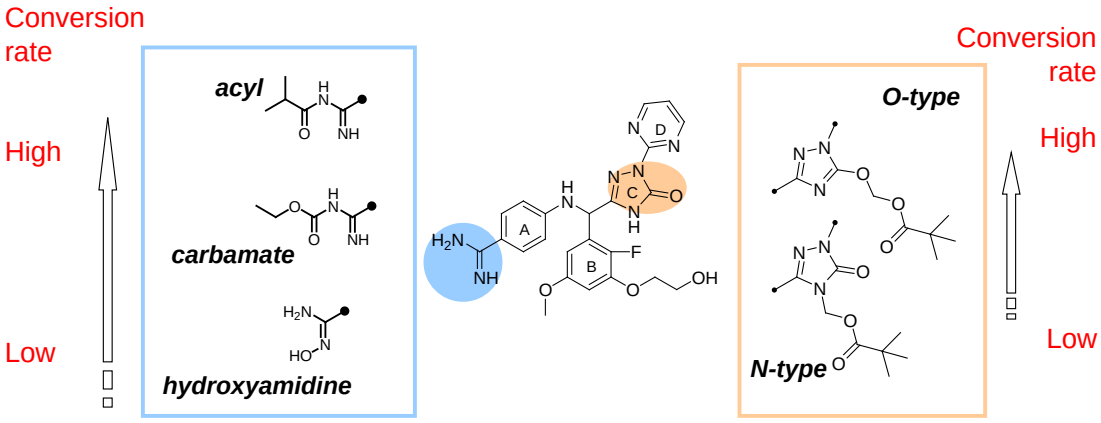


Fig. 6. Design of prodrug form of ER-410660 to improve its oral availability.

This double prodrug formation, or triple prodrug formation with the hydroxyl group also masked, was effective at improving the oral bioavailability of ER-410660. More than one thousand prodrugs of ER-410660 were synthesized and confirmed their oral bioavailability. As a result, three prodrugs were found as attractive compounds for further development. Among these prodrugs, E5539 showed the highest BA in all preclinical animals. The PK profile of E5539 in mouse, rat, and rhesus monkey are summarized in Table 5. Improvement of oral BA by prodrug formation was obvioused in mice, rats and rhesus monkeys at doses of 1-5 mg/kg. To evaluate the intrinsic absorption, a solution containing 2-4 % DMSO and 10 % Tween 20 was used as a dosing vehicle to solve E5539 completely. The BA was calculated by dividing AUC value of ER-410660 after oral dosing of E5539 by that after intravenous (iv) dosing of ER-410660, giving the results 47.2, 3.9 and 17.0 % in mice, rats and rhesus monkeys, respectively. Although the BA in rat was relatively low compared to those in the other two species, a significant improvement due to prodrug formation was observed by E5539 in all species studied.

Table 5: PK parameters of ER-410060 after oral administration of E5539 (A) and comparison of BA values in mice, rats and rhesus monkeys (B)

(A)

Species	Dose (mg/kg)	C_{max} (ng/mL)	T_{max} (hr)	AUC (ng*hr/mL)	BA (%)
Mouse	4.5	530	0.25-0.5	1215	47.2
Rat	1	34	0.5-1	80	3.9
Monkey	5	531	2-4	4400	17.0

(B)

Species	Bioavailability as ER-410660 (%)		Fold increase
	ER-410660	E5539	
Mouse	2.9	47.2	16
Rat	0.8	3.9	4.9
Monkey	2.9	17.0	5.7

E5539 dissolved with 2-4 % DMSO and 10 % Tween 20 was administered to mice, rats and monkeys at the doses of 1-5 mg/kg.

CHAPTER III: In vitro pharmacological assessment of ER-410660

Objective

The *in vitro* enzyme inhibitory profile and anticoagulation selectivity of the TF/VIIa complex inhibitor, ER-410660 were assessed using enzyme inhibitory assay on several coagulation factors and plasma clotting assays.

Material and Methods

Reagents

ER-410660 was synthesized by Tsukuba Research Laboratories, Eisai Co., Ltd. (Ibaraki, Japan). L-α-cephalin, human thrombin, and pancreatic trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human TF was purchased from Calbiochem (La Jolla, CA, USA), and human factors VIIa, IXa, Xa, XIa, and XIIa were purchased from Enzyme Research Laboratories (South Bend, IN, USA). Chromozym TH; Spectrozyme® FVIIa; and Pefachrome® FIXa, FXIIa, and TRY were obtained from Roche (Indianapolis, IN, USA), American Diagnostica (Stamford, CT, USA), and Pentapharm (Basel, Switzerland), respectively. S-2765 was purchased from Daiichi Pure Chemicals (Tokyo, Japan), and 0.02 mol/L $CaCl_2$ was obtained from Sysmex Co. (Hyogo, Japan). Thromborel S, activated cephaloplastin reagent, and thromboplastin C+ were obtained from Dade Behring Marburg GmbH (Marburg, Germany).

Pooled plasma preparation

Rhesus monkeys (n = 27) and Crl:CD (SD) rats (n = 11) were anesthetized by intramuscular administration of a combination of ketamine (5 mg/kg) and xylazine (2

mg/kg) or pentobarbital (50 mg/kg), respectively. Blood, 3 mL from a saphenous vein (rhesus monkey) and 10 mL from the abdominal aorta (rat), was drawn using syringes containing 3.8% sodium citrate, and centrifuged at 1,500 × g (Kubota 8850; Kubota, Tokyo, Japan) for 10 min at room temperature. The resultant plasma was pooled, according to species, and stored at –80°C. Human pooled plasma was purchased from George King Bio-Medical (Overland Park, Kansas, USA).

Enzyme inhibitory assays

Enzyme solutions are shown in Table 6 and were prepared on the day of use. Enzyme assays were performed by incubating a 110 μL sample of each enzyme solution with 15 μL of the various concentrations of ER-410660 (100 mmol/L dissolved in dimethyl sulfoxide (DMSO) and diluted with buffer 1 (100 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1.0 mg/mL BSA; pH 7.4)). Enzyme-specific substrate solution (25 μL of each; final volume = 150 μL) was added to initiate the reaction. The enzymatically released p-nitroanilide was measured by its absorbance at 405 nm. Changes in absorbance were monitored using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). The activities of thrombin, factors IXa, factor Xa, factor XIa, factor XIIa, and trypsin were monitored for 10 min at room temperature, and the activity of TF/VIIa was monitored for 40 min, also at room temperature.

Table 6: Enzyme solutions and substrates

Enzyme Solution	Buffer	Buffer Solution	Final Enzyme Concentration(s)	Substrate
Thrombin	Buffer 1 (pH 7.4)	100 mmol/L Tris-HCl 150 mmol/L NaCl 1.0 mg/mL bovine serum albumin (BSA)	0.10 U/mL	Choromozym TH
TF/VIIa complex	Buffer 2 (pH 7.4)	Buffer 1 15 mmol/L CaCl2 30 μg/mL L-α-cephalin	0.39 μg/mL TF and 0.30 μg/mL VIIa	Spectrozyme FVIIa
Factor IXa	Buffer 3 (pH 7.4)	Buffer 1 40% ethylene glycol	7.0 μg/mL	Pefachrome FIXa
Factor Xa	Buffer 1 (pH 7.4)	100 μμολ/L Tris-HCl 150 mmol/L NaCl 1.0 mg/mL BSA	5.0 mU/mL	S-2765
Factor XIa	Buffer 1 (pH 7.4)	100 mmol/L Tris-HCl 150 mmol/L NaCl 1.0 mg/mL BSA	1.5 μg/mL	Pefachrome FXIa
Factor XIIa	Buffer 1 (pH 7.4)	100 mmol/L Tris-HCl 150 mmol/L NaCl 1.0 mg/mL BSA	1.0 μg/mL	Pefachrome FXIIa
Trypsin	Buffer 1 (pH 7.4)	100 mmol/L Tris-HCl 150 mmol/L NaCl 1.0 mg/mL BSA	1.0 U/mL	Pefachrome TRY

**Chromozym TH; Spectrozyme FVIIa; S-2765; and Pefachrome FIXa, FXIIa, and TRY were each dissolved in purified water to make 1.0 mmol/L solutions. Pefachrome FXIa was dissolved in purified water to make 2.0 mmol/L solutions.*

Plasma Clotting Assay

ER-410660 was dissolved in DMSO to yield a 100 mmol/L concentration, and serially diluted with vehicle (100 mmol/L Tris-HCl and 1.0 mg/mL BSA; pH 7.4). All solutions were prepared on the day of use. Plasma (90 μL) was incubated with the drug solution or vehicle (10 μL) for 2 min at 37°C before clot formation commenced. In the prothrombin time (PT) test, clotting was initiated by adding thromborel S solution (200 μL) to each incubated sample. In the activated partial thromboplastin time (aPTT) test, activated cephaloplastin reagent (100 μL) was incubated with each sample for 1 min at 37°C. $CaCl_2$ solution (100 μL; 0.02 mol/L) was then added to initiate clot formation. Coagulation time was determined using a coagulometer (KC10A, Amelung GmbH, Lemgo, Germany), and the anticoagulant effect was determined using the following formula:

Anticoagulant effect (percentage of control) = A/B × 100, where A = coagulation time of samples with drug and B = coagulation time of control.

The concentration that increased the time taken to reach coagulation by 150% in the PT test and 200% in the aPTT test were defined as PT × 1.5 and aPTT × 2.0, respectively.

Statistical Analyses

All statistical analyses were conducted using the SAS 8.1 or 8.2 software packages (SAS Institute Japan, Tokyo, Japan), and all results are expressed as the means ± standard error of the mean (SEM). For the *in vitro* enzyme assays, the inhibitory constant (Ki) was calculated according to the Cheng–Prusoff equation ($Ki = IC_{50}/1 + [S]/Km$), where [S] is the substrate concentration and Km is the Michaelis–Menten constant. The mean IC_{50} value and the 95% confidence intervals (CI) were determined

based on the IC_{50} values generated from separate sigmoid curves representing the activity of each enzyme versus the ER-410660 concentration from 3 individual experiments. For the *in vitro* coagulation assays, the mean values and the 95% CIs were determined from the PT × 1.5 and aPTT × 2.0 values generated from separate regression lines, representing each anticoagulant activity value versus the ER-410660 concentration of 3 individual experiments.

Results

Inhibitory potency of ER-410660 on TF/VIIa complex and selectivity against other coagulation factors

The Ki values and 95% CIs of ER-410660 for each enzyme are summarized in Table 7. ER-410660 inhibited TF/VIIa activity (Ki value of 0.0049 μmol/L), but only slightly inhibited factors IXa, factor Xa, and factor XIIa, even at a higher concentration (100 μmol/L) than that used to inhibit TF/VIIa activity. The Ki values for thrombin, factor XIa, and trypsin were 1.1, 0.67 and 1.9 μmol/L, respectively, and these values were 224-fold, 137-fold, and 388-fold higher than those of TF/VIIa, respectively.

Table 7: Human protease inhibitory profile of ER-410660

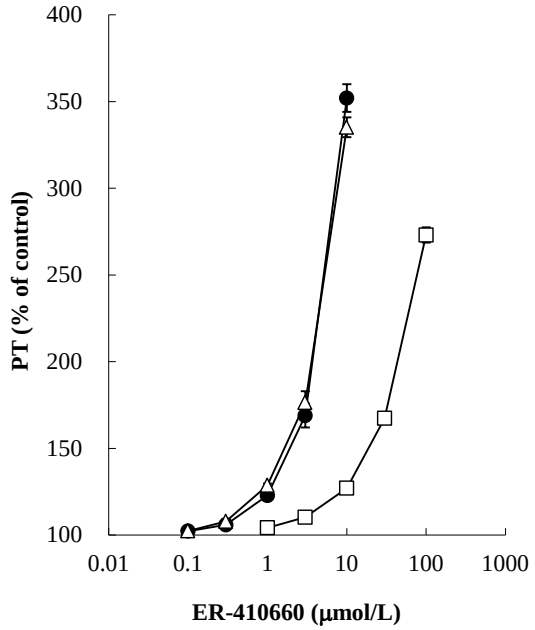
	Ki (μmol/L)						
	Thrombin	**TF/VIIa**	**IXa**	**Xa**	**XIa**	**XIIa**	**Trypsin**
ER-410660	1.1	0.0049	>100	>100	0.67	>100	1.9
	(0.87 - 1.6)	(0.0028 - 0.0083)			(0.36 - 1.3)		(1.1 - 3.2)

Data shown are the means of Ki values and 95% confidential intervals (CI) determined from the results of 3 separate experiments; each performed in triplicate.

Anticoagulation potency of ER-410660 and its pathway selectivity

The relationships between the ER-410660 concentration and the anticoagulant effects on PT and aPTT in human, rhesus monkey, and rat plasma are shown in Fig. 4A-B. ER-410660 increased the PT in human, rhesus monkey, and rat plasma in a concentration-dependent manner. In the PT assay, ER-410660 showed almost equal anticoagulant activity in human and rhesus monkey plasma, but was approximately 10 times less potent against rat plasma. The PT × 1.5 values were 2.0, 1.7, and 19 μmol/L in human, rhesus monkey, and rat plasma, respectively. A much higher concentration of ER-410660 was necessary to double the aPTT in human, rhesus monkey, and rat plasma, and the ratio of aPTT × 2.0 to PT × 1.5 for ER-410660 in human, rhesus monkey, and rat plasma was 25, 18, and 4.6, respectively (Table 8).

(A)



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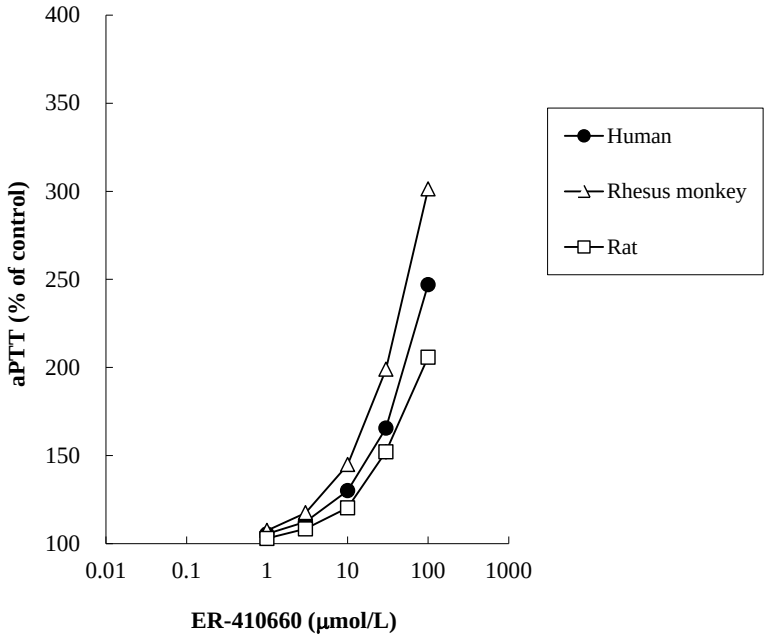


Fig. 4. Anticoagulation potency of ER-410660 in human, rhesus monkey, and rat plasma. The anticoagulation effect at each ER-410660 concentration is shown as a percentage of the control. (A) Prolongation of PT; (B) Prolongation of aPTT. Each data point represents the mean ± SEM from 3 independent experiments, performed in triplicate.

Table 8: Concentration required to prolong coagulation time by 150% in the PT assay (PT × 1.5) and by 200% in the aPTT assay (aPTT × 2.0) in human, rhesus monkey and rat plasma.

	ER-410660 (μmol/L)		
	Human	**Rhesus monkey**	**Rat**
PTx1.5	2.0 (1.1-3.4)	1.7 (1.2-2.4)	19 (18-19)
aPTTx2.0	50 (42-59)	30 (27-34)	88 (67-110)
Selectivity	25	18	4.6

The mean values and the 95% CIs were determined from the PT × 1.5 and aPTT × 2.0 values generated from separate regression lines representing each anticoagulation activity value versus compound concentrations from 3 individual experiments.

Discussion

In the present study, ER-410660 was proved as a potent enzyme inhibitor of the TF/VIIa complex, with a Ki value of 0.0049 μmol/L. Although ER-410660 had a slight inhibitory effect on thrombin and XIa, it was more than 100 times more selective than other coagulation enzymes. In preliminary experiments, ER-410660 also did not show inhibitory activity against tissue plasminogen activator, plasmin, and activated protein C at concentrations of ≤10 μmol/L. ER-410660 selectively prolonged PT, but had little effect on aPTT in human, rhesus monkey, and rat plasma. Theoretically, a TF/VIIa inhibitor should not affect the aPTT assay. The prolonged aPTT, caused by the addition of a high concentration of ER-410660, may be due to its ability to weakly inhibit thrombin and XIa activity. To achieve PTx1.5, ER-410660 needed around 10 times higher concentrations in rat plasma than in human plasma. On the other hand, there are no siginificant species differences between rhesus monkey and human in terms of anticoagulation potency in plasma.

CHAPTER IV: In vivo pharmacological activity of ER-410660 and E5539

Objective

In vivo effects of ER-410660 and E5539 were determined using a TF-induced, thrombin generation rhesus monkey model, a stasis-induced venous thrombosis rat model, a photochemically-induced arterial thrombosis rat model, and a rat tail-cut bleeding model.

Material and Methods

Reagents

ER-410660, E5539, and active comparators, such as dabigatran and rivaroxaban, were synthesized by Tsukuba Research Laboratories, Eisai Co., Ltd. (Ibaraki, Japan). Rose bengal was purchased from Sigma-Aldrich (St. Louis, MO, USA). Thromboplastin C+, and Enzygnost thrombin-antithrombin (TAT) were obtained from Dade Behring Marburg GmbH (Marburg, Germany).

TF-induced, thrombin generation in a rhesus monkey model

Before the beginning of study, Laboratory Animal Care and Use Committee at Eisai Co., Ltd. had approved all of studies plan. These studies were performed in compliance with Laboratory Animal Policy at Eisai. ER-410660 was dissolved in vehicle solution (5% glucose containing 1% DMSO and 0.05 mol/L HCl) to yield 0.06, 0.2, and 0.6 mg/mL solutions. Thromboplastin C+ was dissolved in saline to yield a 100 mU/mL TF solution. All dosing formulations were prepared on the day of use, unless otherwise indicated. The experiment consisted of 4 treatments: vehicle (control), 0.03 mg/kg, 0.1 mg/kg, and 0.3 mg/kg ER-410660. One group of 8 male rhesus monkeys (4–

5-years-old) was used in each of the 4-period crossover experiments. The 4 treatments were administered separately, with at least 7 days washout between compound and vehicle dosing.

The monkeys were anesthetized by intramuscular administration of ketamine (5 mg/kg) and xylazine (2 mg/kg). ER-410660 or vehicle (0.5 mL/kg) was administered intravenously, via the left femoral vein, 2 minutes prior to the TF injection (50 mU/kg in a volume of 0.5 mL/kg) in the right femoral vein. Blood (0.4 mL) was drawn from a cephalic vein 5 minutes later using a syringe with an added 40 μL of 3.8% sodium citrate and centrifuged at 3,000 × g for 5 min at room temperature. *De novo*, TF-induced thrombin generation was determined by measuring the plasma TAT complex levels using an enzyme-linked immunosorbent assay kit (Enzygnost TAT). The absorbance was measured at 492 nm using a microplate spectrophotometer.

Stasis-induced, venous thrombosis rat model

Thromboplastin C+ was dissolved in saline to yield a 4 mU/mL TF solution on the day of use. In the first experiment, ER-410660 was dissolved in the vehicle solution (5% glucose containing 1% DMSO and 0.05 mol/L HCl) to yield 0.1, 0.3, and 1.0 mg/mL solutions. SD rats (males, 10-weeks-old) were randomly allocated to 4 groups (5 animals/group): vehicle (control), 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg ER-410660.

For the second set of experiments, 1, 3, and 10 μg/mL dabigatran solutions and 20, 60, and 200 μg/mL rivaroxaban solutions were prepared, using the same vehicle solution as was used for ER-410660. SD rats were randomly allocated to 7 groups (5 animals/group): vehicle (control); 1 μg/kg, 3 μg/kg, and 10 μg/kg dabigatran; and 20 μg/kg, 60 μg/kg, and 200 μg/kg rivaroxaban.

Rats were weighed and anesthetized by intraperitoneal injection of pentobarbital sodium (55 mg/kg). After surgically opening the abdomen, the branches of the inferior vena cava were ligated. The vehicle or compound solutions were injected via the femoral vein in a volume of 1 mL/kg, followed by 4 mU/kg thromboplastin C+, 30 s later. Venous thrombosis was then induced by tight ligation of the inferior vena cava just below the left renal venous branch, 30 s after the injections. Ten min later, the vena cava was clamped, 1.5 cm from the left renal venous branch, and the vascular segment was longitudinally opened to allow the removal of the thrombus. The thrombus was gently removed and placed on filter paper for 1 min to remove any blood from the surface. It was then transferred into a tube, which was tightly sealed until an accurate weighing could be performed.

Photochemically-induced, arterial thrombosis rat model

As an initial experiment, ER-410660 was dissolved in vehicle (5% glucose) at a concentration of 3 mg/mL, and diluted with vehicle to yield 1 and 0.3 mg/mL solutions. SD rats (males, 10-weeks-old) were randomly allocated to 4 groups (8 animals/group): vehicle (control), 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg ER-410660.

For the second set of experiments, 0.03, 0.1, and 0.3 mg/mL dabigatran solutions and 0.1, 0.3, and 1.0 mg/L rivaroxaban solutions, in 5% glucose, were prepared. SD rats were randomly allocated to 7 groups (5 animals/group): vehicle (control); 0.03 mg/kg, 0.1 mg/kg, and 0.3 mg/kg dabigatran; and 0.1 mg/kg, 0.3mg/kg, and 1.0 mg/kg rivaroxaban.

The rats were weighed and anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The left femoral vein was exposed to inject compound, vehicle, or

rose bengal solutions. The right femoral artery was exposed and the connective tissue around the artery was carefully separated. A pulsed Doppler flow probe (HHD-20, Crystal Biotech, Northborough, MA, USA) was attached to the femoral artery to measure blood flow. Transillumination of green light (540 nm wavelength, 500,000 Lux) was achieved with an irradiation apparatus (L4887, Hamamatsu Photonics, Hamamatsu, Japan). The irradiation was provided through an optical fiber (A5355, Hamamatsu Photonics) positioned 5–10 mm above a femoral artery segment, proximal to the flow probe. Blood flow was measured with the pulsed Doppler flow probe connected to a pulsed Doppler module (PD-1, Valpey-Fisher). Blood flow was continuously recorded on a thermal array recorder (WS-682G, Nihon Kohden, Tokyo, Japan). After establishing the baseline blood flow, irradiation to the femoral artery was initiated. Four min after starting the irradiation, ER-410660, at doses of 0.3, 1, and 3 mg/kg, or vehicle was injected intravenously into the left femoral vein at a volume of 1 mL/kg, followed by 10 mg/kg of rose bengal 1 min later.

The time required to occlude the femoral artery after the injection of rose bengal was measured. Cessation of femoral blood flow was judged by the monitor sounds of the Doppler meter. The artery was considered occluded when the blood flow was completely stopped for > 2 min. Irradiation was continued until either the artery was completely occluded or up to a maximal observation time of 30 min. The time to occlusion (TTO) was determined by measuring the time from the injection of rose bengal to the complete cessation of blood flow, using a chart recorder with a vernier caliper set to ‘mm’. The chart speed of the thermal recorder was 10 mm/min, and the TTO was presented as ‘min’.

Rat ex vivo coagulation assay after intravenous administration

ER-410660 was dissolved in 5% glucose at a concentration of 1 mg/mL. SD rats (males, 10-weeks-old, n = 3) were weighed and anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The left femoral vein was exposed to inject the compound. Blood (0.45 mL) was drawn from a jugular vein before, and 1, 5, and 15 min after drug administration, using a syringe containing 0.05 mL of 3.8% sodium citrate. Blood was centrifuged at 1,500 × g for 5 min at room temperature, and prepared plasma was used for the coagulation time (PT and aPTT) measurements.

Rat tail-cut bleeding model

ER-410660 was dissolved in 5% glucose at a concentration of 7.5 mg/mL, and further diluted with vehicle to yield 5.0 and 2.5 mg/mL solutions. SD rats (males, 10-weeks-old) were randomly allocated to 4 groups (8 animals/group): vehicle (control), 2.5 mg/kg, 5.0 mg/kg, and 7.5 mg/kg ER-410660.

For the second set of experiments, 0.03, 0.1, and 0.3 mg/mL of dabigatran and 0.02, 0.06, 0.2, and 0.6 mg/mL of rivaroxaban solutions, in 5% glucose, were prepared. SD rats were randomly allocated to 8 groups (6 animals/group): vehicle (control); 0.03 mg/kg, 0.1 mg/kg, and 0.3 mg/kg dabigatran; and 0.02 mg/kg, 0.06 mg/kg, 0.2 mg/kg, and 0.6 mg/kg rivaroxaban.

Rats were weighed and then anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The vehicle or compound solutions were injected into the femoral vein at a volume of 1 mL/kg. After 1 min, the tail of the rat was transected 1 mm from the tip and immersed into 37°C saline. Bleeding was observed for 30 min. Complete

hemostasis was defined as the time point at which the bleeding had completely stopped for 5 min. In these cases, the experiment was terminated before the 30 min observation period.

Pharmacological evaluation of prodrug E5539 in a TF-induced, thrombin generation rhesus monkey model

E5539 was dissolved in vehicle (distilled water containing 10% Tween 20) at a concentration of 15 mg/mL and further diluted with vehicle to yield 5 mg/mL and 1.5 mg/mL solutions. The dosing formulations were prepared on the first day of each experimental period. Thromboplastin C+ was dissolved in saline to yield a 100 mU/mL TF solution on the day of use. One group of 11 male rhesus monkeys was used in each of the 4-period crossover experiments. The 4 treatments were administered separately, with at least a 7-day washout period between compound and vehicle dosing.

E5539 at doses of 3, 10 and 30 mg/kg, or vehicle, was orally administered in a 2 mL/kg volume to the male rhesus monkeys. Blood (0.9 mL) was drawn from a brachial vein 3 h after the drug administration using a syringe containing 0.1 mL of 3.8% sodium citrate. TF solution (50 mU/kg) was then administered in a 0.5 mL/kg volume, via the right femoral vein, and blood (0.45 mL) was drawn from a brachial vein 5 min later, using a syringe containing 0.05 mL of 3.8% sodium citrate. The blood was centrifuged at 1,500 × g for 5 min at room temperature, and the prepared plasma was stored at –80°C before coagulation time or TAT complex measurements. *De novo*, TF-induced thrombin generation was determined by measuring the plasma TAT levels using an Enzygnost TAT assay.

Statistical Analyses

All statistical analyses were conducted using the SAS 8.1 or 8.2 software packages (SAS Institute Japan, Tokyo, Japan), and all results are expressed as the means ± standard error of the mean (SEM). For the *in vivo* models, ED_{50} values (the dose causing a 50% reduction relative to controls), TTO_2 values (the dose required to double the TTO of the control group), and BT_2 values (the dose required to double the total bleeding time of the control group) were calculated using linear regression with 95% CIs. Coagulation time experiments were performed in duplicate, and TAT levels were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A probability (p) value of < 0.05 (two-sided) was considered statistically significant.

Results

Inhibitory effect of ER-410660 on TF-induced thrombin generation in the rhesus monkey

ER-410660 decreased *de novo* thrombin generation and reduced the size of the thrombi in a dose-dependent manner. *De novo* thrombin generation was induced by injection of the thromboplastin reagent in a rhesus monkey model and measured using plasma TAT levels. The plasma TAT level in the control group was 314 ± 37 ng/mL, and intravenous administration of ER-410660 decreased this level, with an ED_{50} value of 0.040 mg/kg (95% CI, 0.025–0.055) (Fig. 5).

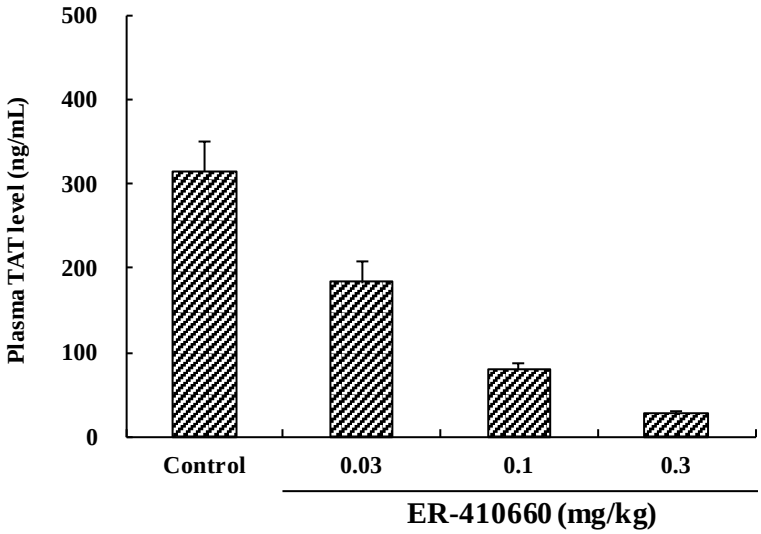
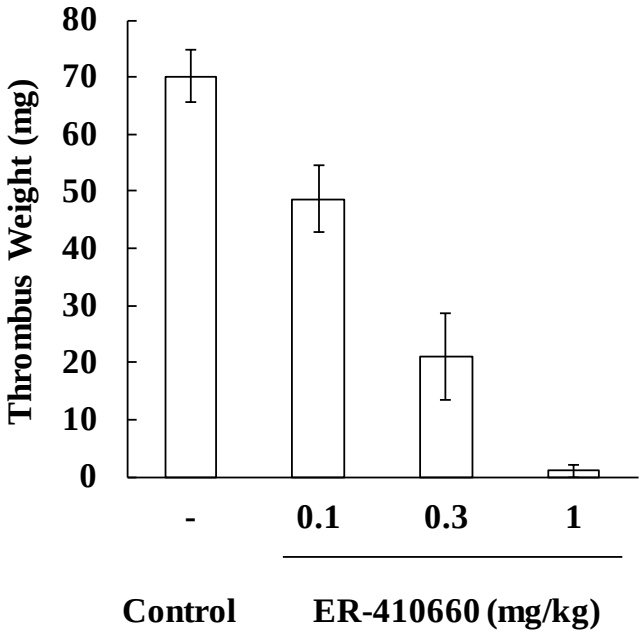


Fig. 5. Effects of ER-410660 on tissue factor (TF)-induced, thrombin-antithrombin (TAT) complex formation in rhesus monkey. Results are expressed as the mean ± SEM; n = 8 animals per group.

Effect of ER-410660 on venous thrombus formation in rats

In the rat model, ER-410660 reduced venous thrombus weight, with an ED_{50} value of 0.18 mg/kg (95% CI, 0.11–0.25) (Fig. 6A) in a stasis-induced venous thrombosis rat model, where the mean wet weight of the thrombi in the control group was 70.1 ± 4.6 mg. An anti-venous thrombotic effect for dabigatran and rivaroxaban was also confirmed in the rat model, with ED_{50} values of 0.0033 (95% CI, 0.0028–0.0039) and 0.074 mg/kg (95% CI, 0.056–0.097), respectively (Fig. 6B).

(A)



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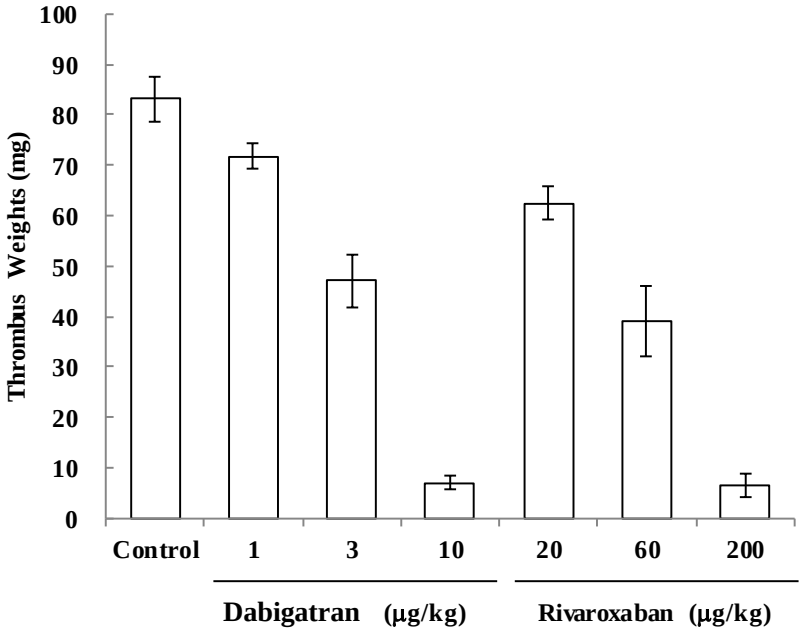
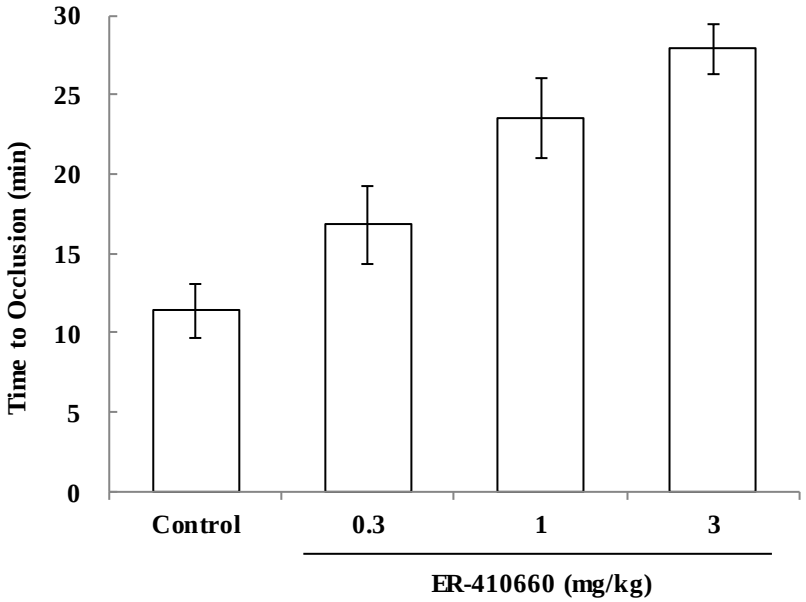


Fig. 6. Effect of ER-410660, dabigatran and rivaroxaban on venous thrombus weights in rat abdominal veins. (A) ER-410660 and (B) dabigatran and rivaroxaban. Results are expressed as the mean ± SEM; n = 5 animals/group.

Antithrombotic effect of ER-410660 in the photochemically-induced arterial thrombosis rat model

The antithrombotic effect of ER-410660 in the photochemically-induced arterial thrombosis rat model is shown in Fig. 7A. ER-410660 caused a dose-dependent prolongation of TTO from 11.4 ± 1.7 min following vehicle administration to 16.8 ± 2.5, 23.5 ± 2.5, and 27.9 ± 1.6 min following administration of 0.3, 1, and 3 mg/kg, respectively. The TTO_2 value was 0.99 mg/kg (95% CI, 0.51–1.9). Dabigatran and rivaroxaban also prolonged TTO in a dose-dependent manner, with TTO_2 values of 0.078 mg/kg (95% CI, 0.047– 0.12) and 0.34 mg/kg (95% CI, 0.24– 0.50), respectively (Fig. 7B).

(A)



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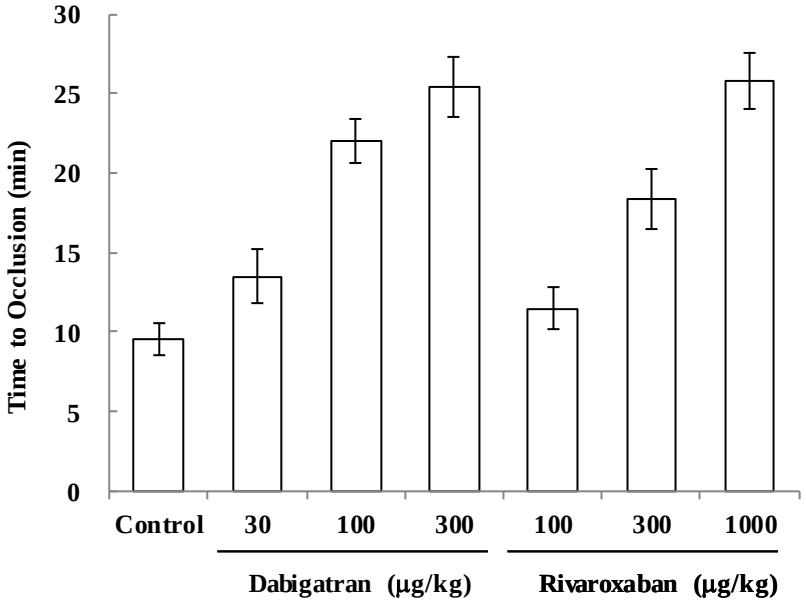


Fig. 7. Effects of ER-410660, dabigatran, and rivaroxaban on time to occlusion in a photochemically-induced, arterial thrombosis rat model. (A) ER-410660 and (B) dabigatran and rivaroxaban. Results are expressed as the mean ± SEM; n = 8 animals/group.

Rat ex vivo coagulation assay after intravenous administration of ER-410660

Coagulation time was measured after the intravenous administration of ER-410660 to rats. The PT and aPTT values after administration of 1 mg/kg of ER-410660 are summarized in Table 9. PT was prolonged by 31%, 18%, and 11% at 1, 5, and 15 minutes after ER-410660 administration, respectively, whereas slight aPTT changes were observed only at 1 min after administration.

Table 9: Ex vivo measurement of PT and aPTT after iv ER-410660 (1 mg/kg) administration in rats

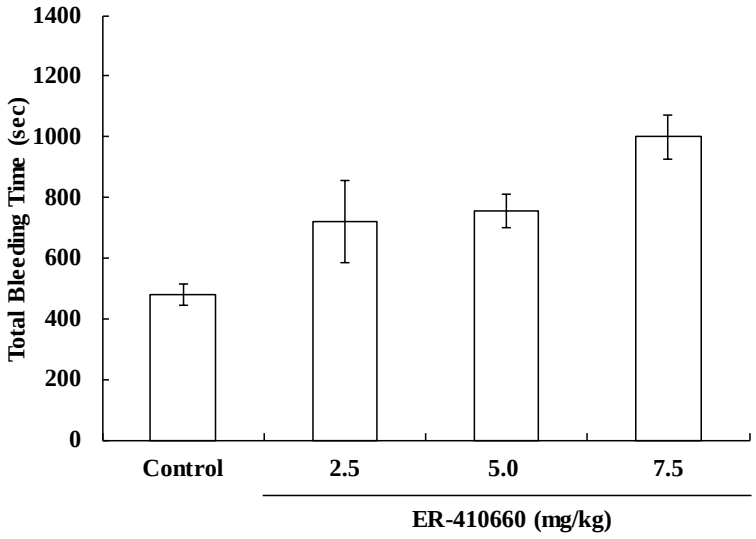
	Coagulation time (sec)				Relative to pre (%)		
	Time after in administration (min)				Time after in administration (min)		
	Pre	**1**	**5**	**15**	**1**	**5**	**15**
PT	10.1 ± 0.2	13.2 ± 0.3	11.9 ± 0.2	11.2 ± 0.3	131 ± 1	118 ± 3	111 ± 3
aPTT	10.8 ± 0.2	12.3 ± 0.4	11.4 ± 0.2	11.1 ± 0.4	113 ± 1	105 ± 1	103 ± 1

Results are expressed as the mean ± SEM; n = 3 animals.

Effect of ER-410660 on bleeding time in the tail-cut model in rats

Since the bleeding time did not differ from the baseline value following intravenous administration of < 1 mg/kg of ER-410660 in the rat tail-cut model, the bleeding risk for ER-410660 was measured at doses (2.5, 5.0, and 7.5 mg/kg) greater than the ED_{50} found in the rat venous thrombosis model. ER-410660 increased the total bleeding time by approximately 1.5-, 1.6-, and 2.1-fold, respectively, in a dose-dependent manner. The calculated BT_2 value was 7.1 mg/kg (95% CI, 6.1–11) (Fig. 8A). Dabigatran and rivaroxaban also increased the total bleeding time in a dose-dependent manner, with BT_2 values of 0.053 mg/kg (95% CI, 0.041–0.071) and 0.060 mg/kg (95% CI, 0.025–0.11), respectively (Fig. 8B).

(A)



(B)

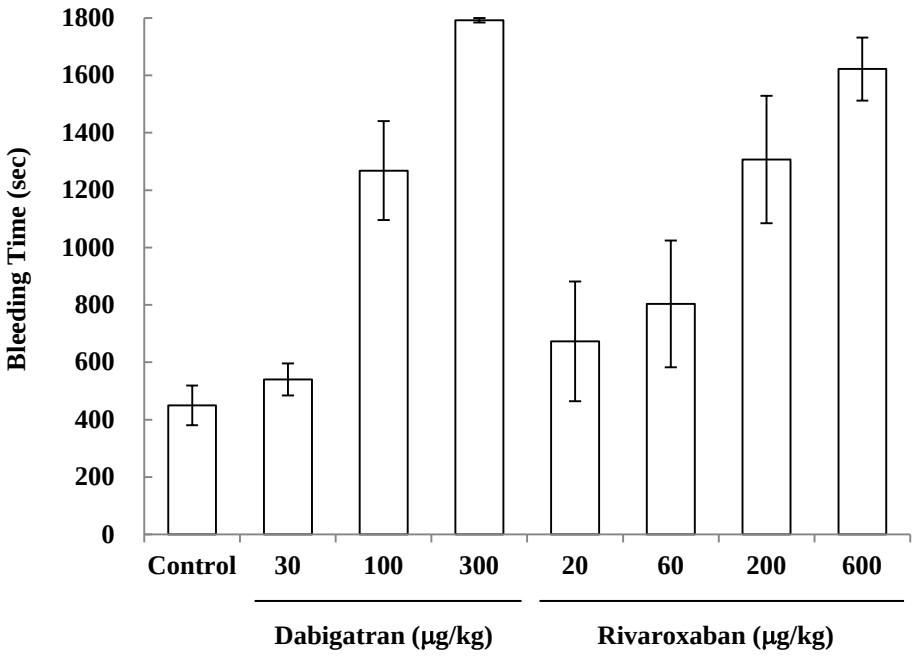


Fig. 8. Effect of ER-410660, dabigatran, and rivaroxaban on total bleeding time in a rat tail-cut model. (A) ER-410660 and (B) dabigatran and rivaroxaban. Results are expressed as the mean ± SEM; n = 8 animals/group.

Inhibitory effects of the oral administration of E5539 on TF-induced thrombin generation

To increase oral availability, drug-discovery efforts focused on prodrug formation identified E5539. The permeated E5539 from intestine was activated by carboxyl esterases and the ER-410660 was released to plasma. The inhibitory effects of the oral administration of E5539 on TF-induced, *de novo* thrombin generation were examined in rhesus monkeys, which have a VIIa structure closely resembling that of humans. PTs and aPTTs, after oral E5539 administration, are summarized in Table 10. Orally administered E5539 prolonged the PT by 16% and 22% at doses of 10 and 30 mg/kg, respectively; aPTT remained unchanged at all doses. Plasma TAT complex levels were reduced in a dose-dependent manner, and statistical significances were observed even at the lowest dose of 3 mg/kg (Fig. 9). The ED_{50} value, which is the dose that causes a 50% reduction of vehicle control TAT levels, was 11.6 mg/kg (95% CI, 8.01−16.7) following oral E5539 administration.

Table 10: Ex vivo measurement of PT and aPTT after oral E5539 administration in rhesus monkey

	Coagulation Time (s)	
Treatment	PT	aPTT
Vehicle	15.2 ± 0.3	25.8 ± 0.8
3 mg/kg	15.4 ± 0.5	24.9 ± 0.7
10 mg/kg	17.6 ± 0.8	26.5 ± 0.8
30 mg/kg	18.6 ± 0.8	26.9 ± 1.0

Results are expressed as the mean ± SEM; n = 11 animals per group.

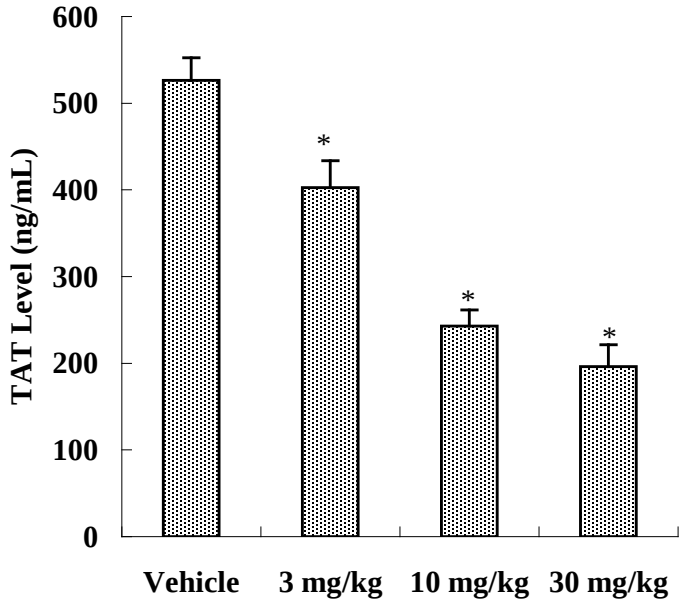


Fig. 9. Effects of oral administration of E5539 on TF-induced TAT complex formation in rhesus monkeys. Results are expressed as the mean ± SEM; n = 11 animals/group (*$p < 0.05$ versus vehicle-treated group).

Discussion

In the experiments examining the *ex vivo* coagulation time, after the intravenous administration of ER-410660 (1 mg/kg), the prepared plasma, 1 min after injection, showed a 31% prolongation of PT compared to the pre-treated plasma, and the aPTT was prolonged by 13%. Because aPTT is more sensitive than the PT to thrombin inhibitors [44], and factor XIa inhibition is theoretically affected only by aPTT, both thrombin and factor XIa inhibition have been suggested to cause dominant prolongation of aPTT. The data showed that PT was dominantly prolonged after the administration of 1 mg/kg ER-410660 and the effectiveness in the venous thrombosis model was shown in less than 1 mg/kg. It was suggested that thrombin and XIa inhibition might not affect in the venous thrombosis models, but it was uncertain whether antithrombotic effects were solely by TF/VIIa inhibition or no when the rats were dosed more than 1 mg/kg or higher.

ER-410660 also doubled the total bleeding time in a rat tail-cut model at a dose of 7.1 mg/kg, which was 39-fold higher than the ED_{50} value in the venous thrombosis model and 7.2-fold higher than the TTO_2 value in the arterial thrombosis model. Table 11 summarizes the calculated bleeding risk of ER-410660, dabigatran, and rivaroxaban. The results show that the TF/VIIa inhibitor has the lowest bleeding propensity in the rat experiments compared to the approved agents that directly inhibit thrombin and factor Xa. The half-life of ER-410660 in rats, after intravenous administration was 1.2 h, compared to the 1.0-h and 0.43-h half-lives of dabigatran and rivaroxaban, respectively (Data not shown). Based on these data, the pharmacokinetic differences did not increase the margin of ER-410660 for bleeding.

Table 11: Summary of in vivo experiments and calculated safety margin of ER-410660, dabigatran, and rivaroxaban against bleeding in rats

	ER-410660	**Dabigatran**	**Rivaroxaban**
Venous thrombosis ED_{50}	0.18 mg/kg	0.0033 mg/kg	0.074 mg/kg
Arterial thrombosis TTO_2	0.99 mg/kg	0.078 mg/kg	0.34 mg/kg
Tail-cut BT_2	7.1 mg/kg	0.053 mg/kg	0.060 mg/kg
Margin 1 (BT_2/ED_{50})	39	16	0.81
Margin 2 (TTO_2/ED_{50})	7.2	0.68	0.18

Orally administered E5539, a prodrug form of ER-410660, also inhibited TF-induced, *de novo* thrombin generation in a rhesus monkey model with an ED_{50} value of 11.6 mg/kg and reduced the plasma TAT complex levels that were elevated by TF-injection. *Ex vivo* coagulation tests showed that the PT for the E5539-administered group (10 mg/kg) was prolonged by 16% compared to that for the vehicle-treated group. In contrast, no changes in aPTT were detected at any E5539 dose. Pharmacological activity has not been previously described in non-human primates for an orally available TF/VIIa inhibitor. Thus, E5539 will promote the development of novel oral anticoagulants that may act as preventive agents for several thrombotic conditions, without also enhancing the risk of bleeding.

CHAPTER V: Discussion

The increased risk of bleeding is a serious complication for almost all antiplatelet, anticoagulant, and fibrinolytic drugs. Direct thrombin and factor Xa inhibitors have an antithrombotic effect, however, their efficacies are associated with a substantial increase in bleeding propensity [14–19]. TF/VIIa complex is known to produce factor IXa at approximately the same rate as factor Xa when both the substrates are present [24]. Thus, sequential activation of coagulation factors IX and X by proteolysis results in an initiation of extrinsic coagulation. This produces an agents that can regulate the cascade's initiation phase by inhibiting TF/VIIa complex that could preserve the intrinsic coagulation pathway, thereby decreasing the bleeding risk. Studies using recombinant VIIa and anti-TF antibody have shown that undesirable changes in hemostatic function, such as bleeding, were not observed in clinical trials, except for the expected prolonging of PT [25,26].

Another recent study has shown that direct inhibition of the TF/FVIIa catalytic complex by recombinant nematode anticoagulant protein c2 (rNAPc2) administered within 1 hour of surgery at a dosage of 3.0 μg/kg yielded the lowest overall venous thrombosis rates with no significant increase in bleeding [27]. These findings were the first evidence, in humans, that a TF/VIIa inhibitor was efficacious for the prevention of venous thrombosis. These findings are the first evidence in humans that a TF/FVIIa inhibitor is efficacious for prevention of venous thrombosis, providing confirmation that suppression of the extrinsic coagulation pathway, which is triggered by TF expression, could be used for prevention of thrombotic events. In this previous study, PT was approximately twice the length of the that observed in the placebo-treated group, while no significant change in the coagulation time of aPTT was observed [27]. These results

indicate that PT should be established as a pharmacodynamics marker, which will allow selection of a suitable clinical dose for agents that selectively inhibit the TF/FVIIa complex.

In the present study, ER-410660 was identified as a potent and selective enzyme inhibitor of the TF/VIIa complex and E5539 as an orally available prodrug form. ER-41066 surely prolongs the extrinsic coagulation pathway and has less anticoagulation efficacy against the intrinsic pathway. ER-410660 showed a greater margin between the BT_2 in the rat tail-cut bleeding model and the ED_{50} in the venous thrombosis model or the TTO_2 in the arterial thrombosis model, indicating that the bleeding risk associated with this TF/VIIa inhibitor is much smaller than that of currently marketed drugs, such as direct thrombin and Xa inhibitors. Moreover, in a series of synthesized compounds, I found that the *in vivo* BT_2 and ED_{50} margins were positively correlated with the selectivity of the anti-coagulation effect on PT [Hirota S et al., 242nd ACS National Meeting]. Taken together, these data further support the attractiveness of a TF/VIIa inhibitor for use as an antithrombotic drug.

Recently, the efficacy and safety of new-generation, oral anticoagulant agents were compared with placebo in patients receiving antiplatelet therapy after acute coronary syndrome (ACS). The pooled results showed that the use of thrombin or Xa inhibitors moderately reduced the risk for stent thrombosis or composite ischemic events, without a significant effect on overall mortality. However, the thrombin and Xa inhibitors were also associated with a dramatic increase in major bleeding events, and treatment with new-generation oral anticoagulant agents did not provide any advantage over placebo on the net clinical benefit [45]. Due to our finding of a low bleeding risk associated with TF/VIIa inhibition, I speculate that targeting the TF/VIIa complex may be a superior

approach for the treatment of not only venous thrombosis, but also atherothrombotic diseases.

The bleeding risk associated TF/VIIa inhibitor should be investigated more thoroughly given that data from gene knockout studies, in mice, have shown that TF, as well as factor VII, factor X, and prothrombin, are essential for hemostasis and embryonic development [46]. Mice that express low levels of TF (low-TF) and low levels of murine factor VII (low-FVII) showed fatal hemorrhaging in the brain, lung, intestine, and uterus. Both types of mice also exhibited hemosiderin deposition and fibrosis in the hearts [5, 47]. In addition, Snyder et al. reported that the administration of human TF monoclonal antibody to human TF knock-in mice caused tissue-specific hemorrhaging in the brain, heart, lung, and testis [48]. Humans with factor VII deficiency are found at a low frequency (1 in 500,000) and these patients experience intra-articular and mucocutaneous bleeding, analogous to patients with hemophilia A or B [49]. Moreover, severe factor VII deficiency is associated with cerebral hemorrhage and perinatal death [50]. On the other hand, a 7-day intravenous administration (ER-410660 at 25 mg/kg) study in rats showed that critical hemorrhaging and abnormalities were not evident in clinical pathology (Data not shown). Thus, the actual bleeding risk of TF/VIIa inhibitors should be elucidated in a future clinical trial.

In addition to its primary role as an initiator of coagulation, the TF/VIIa complex is also known to influence protease-activated, receptor-dependent tumor cell behaviors, such as proliferation, metastasis, and angiogenesis, suggesting that TF may also determine tumor progression. A strong correlation between cancer and aberrant hemostasis is now widely recognized, and patients with various types of cancer, including pancreatic, colorectal, and gastric cancers often develop thrombosis [51].

These findings underline the importance of developing orally active TF/VIIa inhibitors, not only to prevent venous thrombosis and other TF-mediated diseases, including arterial thrombotic events, intracardiac thrombus formation, sickle cell anemia, and inflammatory disorders [46], but also for their potential use as oncology-related drugs [46,49]. Having both parenteral and orally available forms of at least one TF/VIIa inhibitor will facilitate the design of specific anti-thrombotic and anti-cancer treatments.

CHAPTER VI: References

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