S cDNA are located on chromosome 3. Thromb Haemost 1987;58:982–987.
8. Dykes AC, Walker ID, McMahon AD, Islam SI, Tait RC. A study of Protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol 2001;113:636–641.
9. Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: The Suita Study. J Thromb Haemost 2004;2:1012–1013.
10. Simioni P, Sanson BJ, Prandoni P, Tormene D, Friederich PW, Girolami B, Gavasso S, Huisman MV, Buller HR, Wouter ten Cate J, Girolami A, Prins MH. Incidence of venous thromboembolism in families with inherited thrombophilia. Thromb Haemost 1999;81:198–202.
11. Martinelli I, Mannucci PM, De Stefano V, Taioli E, Rossi V, Crosti F, Paciaroni K, Leone G, Faioni EM. Different risks of thrombosis in four coagulation defects associated with inherited thrombophilia: a study of 150 families. Blood 1998;92:2353–2358.
12. D'Andrea G, Di Perna P, Brancaccio V, Faioni EM, Castaman G, Cibelli G, Di Minno G, Margaglione M. A novel G-to-A mutation in the intron-N of the protein S gene leading to abnormal RNA splicing in a patient with protein S deficiency. Haematologica 2003;88:459–464.
13. Okada H, Takagi A, Murate T, Adachi T, Yamamoto K, Matsushita T, Takamatsu J, Sugita K, Sugimoto M, Yoshioka A, Yamazaki T, Saito H, Kojima T. Identification of protein Sα gene mutations including four novel mutations in eight unrelated patients with protein S deficiency. Br J Haematol 2004;126:219–225.
14. Biguzzi E, Razzari C, Lane DA, Castaman G, Cappellari A, Bucciarelli P, Fontana G, Margaglione M, D'Andrea G, Simmonds RE, Rezende SM, Preston R, Prisco D, Faioni EM. Molecular diversity and thrombotic risk in protein S deficiency: The PROSIT study. Hum Mutat 2005;25:259–269.
15. Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, Tsuda H, Kang D, Hamasaki N. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. Clin Biochem 2005;38:908–915.
16. Gandrille S, Borgel D, Sala N, Espinosa-Parrilla Y, Simmonds R, Rezende S, Lind B, Mannhalter C, Pabinger I, Reitsma PH, Formstone C, Cooper DN, Saito H, Suzuki K, Bernardi F, Aiach M, Ireland H, Lane DA, Espinosa Y. Protein S deficiency: a database of mutations-summary of the first update. Thromb Haemost 2000;84:918.
17. Tatewaki H, Iida H, Nakahara M, Tsuda H, Kinoshita S, Kanaji T, Yoshida N, Miyazaki S, Hamasaki N. A novel splice acceptor site mutation which produces multiple splicing abnormalities resulting in protein S deficiency type I. Thromb Haemost 1999;82:65–71.
18. Hirose M, Kimura F, Wang HQ, Takebayashi K, Kobayashi M, Nakanishi K, Akiyama M, Kimura T, Noda Y. Protein S gene mutation in a young woman with type III protein S deficiency and venous thrombosis during pregnancy. J Thromb Thrombolys 2002;13:85–88.
19. Simmonds RE, Ireland H, Kunz G, Lane DA. Identification of 19 protein S gene mutations in patients with phenotypic protein S deficiency and thrombosis. Protein S Study Group. Blood 1996;88:4195–4204.
20. Iwaki T, Mastushita T, Kobayashi T, Yamamoto Y, Nomura Y, Kagami K, Nakayama T, Sugiura I, Kojima T, Takamatsu J, Kanayama N, Saito H. DNA sequence analysis of protein S deficiency-identification of four point mutations in twelve Japanese subjects. Semin Thromb Hemost 2001;27:155–160.
21. Mustafa S, Pabinger I, Varadi K, Halbmayer WM, Lechner K, Schwarz HP, Fischer M, Mannhalter C. A hitherto unknown splice site defect in the protein S gene (PROS1): the mutation results in allelic exclusion and causes type I and type III protein S deficiency. Br J Haematol 1997;99:298–300.
22. Nakahara M, Iida H, Urata M, Fujise M, Wakiyama M, Kinoshita S, Tsuda H, Okamura T, Yao K, Yao T, Hamasaki N. A novel splice acceptor site mutation of protein S gene in affected individuals with type I protein S deficiency: allelic exclusion of the mutant gene. Thromb Res 2001;101:387–393.
23. Iida H, Nakahara M, Komori K, Fujise M, Wakiyama M, Urata M, Kinoshita S, Tsuda H, Sugimachi K, Hamasaki N. Failure in the detection of aberrant mRNA from the heterozygotic splice site mutant allele for protein S in a patient with protein S deficiency. Thromb Res 2001;102:187–196.
24. Stenflo J, Stenberg Y, Muranyi A. Calcium-binding EGF-like modules in coagulation proteinases: function of the calcium ion in module interactions. Biochim Biophys Acta 2000;1477:51–63.
25. Muranyi A, Evenas J, Stenberg Y, Stenflo J, Drakenberg T. Characterization of the EGF-like module pair 3–4 from vitamin K-dependent protein S using NMR spectroscopy reveals dynamics on three separate time scales and extensive effects from calcium binding. Biochemistry 2000;39:15742–15756.
26. Kurniawan ND, O'Leary JM, Thamlitz AM, Sofair R, Werner JM, Stenflo J, Downing AK. N-Terminal domain linkage modulates the folding properties of protein S epidermal growth factor-like modules. Biochemistry 2004;43:9352–9360.
27. Drakenberg T, Ghasriani H, Thulin E, Thamlitz AM, Muranyi A, Annila A, Stenflo J. Solution structure of the $Ca^{2+}$-binding EGF3-4 pair from vitamin K-dependent protein S: Identification of an unusual fold in EGF3. Biochemistry 2005;44:8782–8789.
28. Sitia R, Braakman I. Quality control in the endoplasmic reticulum protein factory. Nature 2003;426:891–894.
29. Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003;426:895–899.
30. Andersen BD, Bisgaard ML, Lind B, Philips M, Villoutreix B, Thorsen S. Characterization and structural impact of five novel PROS1 mutations in eleven protein S-deficient families. Thromb Haemost 2001;86:1392–1399.
31. Villoutreix BO, Dahlback B, Borgel D, Gandrille S, Muller YA. Three-dimensional model of the SHBG-like region of anticoagulant protein S: new structure-function insights. Proteins 2001;43:203–216.

[Frontiers in Bioscience 11, 232-248, January 1, 2006]

# Factor Xa inhibitors: new anti-thrombotic agents and their characteristics

Masahiro Ieko [1], Takashi Tarumi.[1], Toru Nakabayashi [1], Mika Yoshida [2], Sumiyoshi Naito [2], and Takao Koike [3]

*[1] Department of Internal Medicine, [2] Department of Clinical Laboratory, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, [3] Department of Medicine II, Hokkaido University School of Medicine, Sapporo, Japan*

TABLE OF CONTENTS



## 1. ABSTRACT

Factor Xa (FXa) is a key enzyme that is positioned at the convergence of the intrinsic and extrinsic pathways in the blood coagulation cascade, and inactivation by a specific FXa inhibitor effectively prevents the generation of thrombin. Various types of low molecular weight (LMW) heparin, which function as semi-selective and indirect FXa inhibitors, are replacing unfractionated heparin (UFH) as agents for the prevention and treatment of venous thromboembolism (VTE), as well as in initial treatment for coronary events. Of those, heparinoid has been shown to be safer and more effective for the prevention of postoperative VTE than UFH, especially for treatment of heparin-induced thrombocytopenia (HIT). Further, synthetic pentasaccharide has been found to offer advantages over current thromboprophylactic regimens in a number of patients undergoing major orthopedic surgery. Other studies have shown that pentasaccharide is more effective for overall VTE in comparison with LMW heparin, though it was also associated with an increased rate of major bleeding. Synthetic, selective, and direct inhibitors to FXa, such as DX-9065a, are highly potent and orally bioavailable antithrombotic agents that have demonstrated an improved side effect profile, probably by allowing sufficient thrombin to remain for platelet activation and normal hemostasis, while preventing pathological thrombus formation. For thrombosis therapy, the most desirable type of antithrombotic agent is an orally active drug that has a broad range of effective doses and no hemorrhagic side effects. Presently, many types of direct inhibitors are in various stages of clinical trials and expected to provide significant benefits as compared to currently utilized therapy strategies.

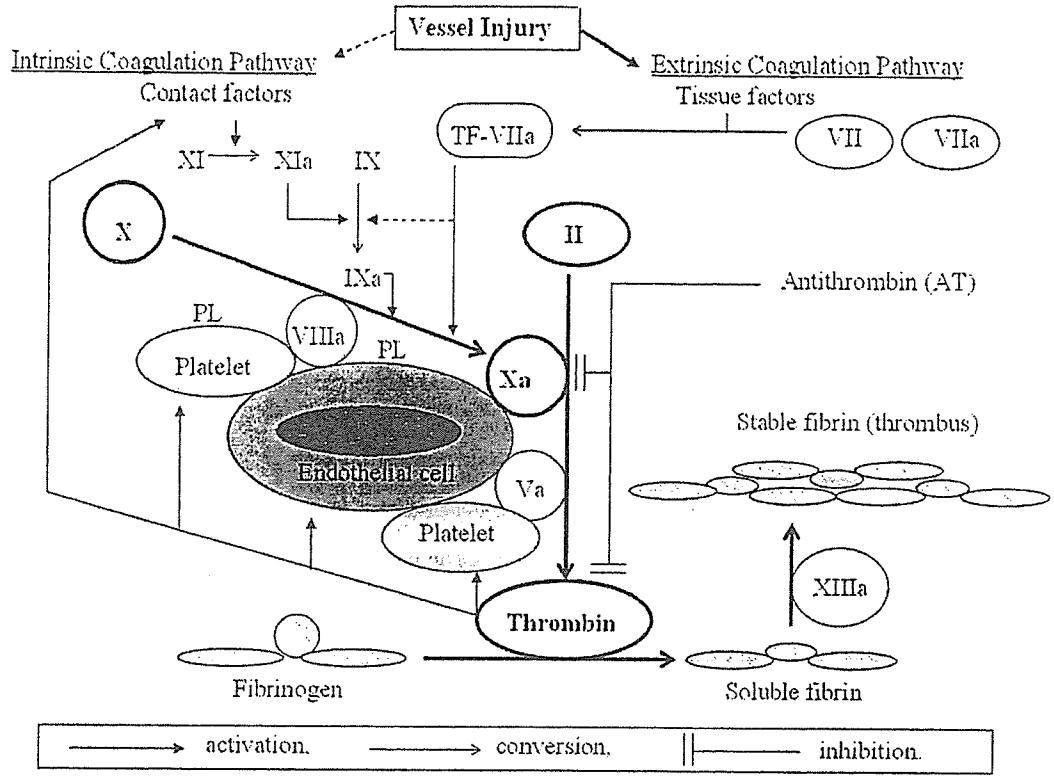


Figure 1. The coagulation cascade.

## 2. INTRODUCTION

Venous and arterial thromboembolism together represent a major public health concern throughout the world, due to the associated morbidity and mortality. Hemostasis is the blood clotting process that occurs when a vasculature injury leads to a series of vasculomotor and cellular reactions, as well as activation of the blood coagulation cascade. That cascade is initiated via an extrinsic pathway, and leads first to thrombin activation and then massively amplified thrombin activation due to positive feedback from the intrinsic pathway (1). Both pathways merge at the factor X activation step, at which time the generated thrombin activates both platelets and endothelial cells, resulting in the expression of negatively charged phospholipids on the cell surfaces, which provides space for the activation of coagulation factors, and in the release of coagulation factors such as fibrinogen and platelet activating factors from those cells, which promotes additional activation of the coagulation system (Figure 1). An imbalance among these clotting processes and thrombolytic conditions can lead to thrombotic or bleeding disorders.

Thromboembolism is responsible for much of the morbidity and mortality seen in clinical practice, and the prevention of blood coagulation is of primary importance in a variety of pathological situations. In the acute phase of thromboembolism, fibrinolytic therapy with a tissue-plasminogen activator is usually performed, whereas antithrombotic agents or anti-platelet agents are employed for prevention of the disease. Both treatment and prevention of thromboembolic disease require an effective anticoagulant therapy without exposing the patient to an increased risk of bleeding, though all presently employed antithrombotic agents have more and less a bleeding tendency as a side effect. Thus, effective monitoring is critical to determine the most effective dose. For example, the measurement of prothrombin time (PT) and the International Normalized Ratio (INR) are utilized worldwide to monitor the antithrombotic effect of warfarin therapy. Since an overdose of an antithrombotic agent will lead to a hemorrhage tendency, a dosage at less than the effective limit will cause thrombosis. Thus, the development of antithrombotic agents with both a broad effective dose and few hemorrhagic side effects is an important pursuit.

The many kinds of FXa inhibitors presently available, including those still in development, are shown in Table 1. Unfractionated heparin (UFH) is a frequently used and well known anticoagulant agent, however, it is an unselective and indirect inhibitor of FXa, and also inhibits thrombin activity in the presence of antithrombin (AT). Various types of low molecular weight (LMW) heparin are administered for thrombotic disorders in many different kinds of clinical cases. Of those, heparinoid and synthetic pentasaccharide have been extensively studied for their biochemical characteristics and basic influence on the coagulation system, while their advantages in clinical use have also been discussed. On the other hand, there have been few clinical trials of synthetic, selective, and direct inhibitors of FXa, though many have been developed and investigated in basic research studies.

Table 1. Classification of factor Xa inhibitors

| | Antithrombotic activity (dependence on antithrombin) | FXa selectivity |
|---|---|---|
| 1. Unfractionated heparin | Indirect | Non-Selective |
| 2. Low molecular weight heparin<br>(1) enoxaparin<br>(2) dalteparin sodium<br>(3) reviparin sodium | Indirect | Semi-Selective |
| 3. Heparinoid<br>danaparoid sodium | Indirect | Semi-Selective |
| 4. Synthetic pentasaccharide<br>(1) fondaparinux<br>(2) Sanorg-34006 | Indirect | Selective |
| 5. Synthetic factor Xa inhibitor | Direct | Selective |

Herein, we review the results of recent basic studies as well as clinical reports of FXa inhibitors, and focus on recent progress toward the development of orally bioavailable agents.

## 3. CLASSIFICATION OF FACTOR Xa INHIBITORS

By virtue of its position at the juncture of the extrinsic and intrinsic pathways of coagulation (2), FXa is considered by many researchers to be an ideal point of intervention for antithrombotic therapy and many of the new synthetic anticoagulants target FXa. Further, extensive preclinical and clinical studies have demonstrated that inhibition of FXa is effective for both venous and arterial thromboembolism (3-5). Four types of inhibitors of FXa have been developed thus far, heparin, heparinoid, synthetic pentasaccharide, and synthetic direct inhibitors (Table 1).

## 4. INDIRECT (ANTITHROMBIN-DEPENDENT) FACTOR Xa INHIBITORS

### 4.1. Low molecular weight heparin

LMW heparin is derived from standard heparin (UFH) through either chemical or enzymatic depolymerization. UFH has a molecular weight of 5,000 to 30,000 daltons, whereas LMW heparin ranges from 1,000 to 10,000 daltons, resulting in properties that are distinct from those of traditional heparin, as it binds less strongly to protein, shows enhanced bioavailability, interacts less with platelets, and has a very predictable dose response, eliminating the need to monitor the activated partial prothrombin time (aPTT) (6). LMW heparin, like UFH, binds to AT, however, it inhibits thrombin to a lesser degree than UFH. Thus, LMW heparin is a semi-selective, indirect inhibitor of FXa. Its clinical advantages include predictability, dose-dependent plasma levels, a long half-life over UFH, and less bleeding for a given antithrombotic effect (7).

Heparin-induced thrombocytopenia (HIT) is a rare but severe complication of heparin therapy caused by antibodies directed against the complexes of platelet factor 4 and heparin. The risk of developing HIT during LMW heparin therapy in patients undergoing orthopedic surgery has been estimated to be 0.75%, in contrast to approximately 5% for patients receiving UFH (8).

#### 4.1.1. Effects of LMW heparin for treatment of coronary events

Clinical trials have been used to evaluate the safety and efficacy of different types of LMW heparin for the reduction of coronary events. In the dalteparin (Fragmin®) study titled Fragmin during instability in coronary artery disease (FRISC), which included 1506 patients with unstable angina or non-Q-wave myocardial infarction, 120 IU/kg of body weight of the agent twice a day for 6 days then at 7500 IU once daily for the next 35-45 days was more effective than a placebo in reducing the incidence of death or myocardial infarction, the double composite endpoint of the study (9). However, no differences were observed between dalteparin administered subcutaneously twice a day and a continuous infusion of UFH in FRIC study (10). Following those trials, a double-blind, placebo-controlled study [Efficacy and Safety of Subcutaneous Enoxaparin in Non-Q-Wave Coronary Events (ESSENCE) trial] was conducted with 3171 patients with angina at rest or non-Q-wave myocardial infarction, for whom 1 mg/kg of enoxaparin (Lovenox®, Clexane®) was administered subcutaneously twice daily. The regimen significantly reduced the incidence of death, myocardial infarction, or recurrent angina relative to UFH, the composite endpoint of the study, however, the incidence of bleeding overall was significantly higher in the enoxaparin group (11). In another study, the safety and efficacy of enoxaparin was evaluated in randomly chosen patients with high-risk non-ST segment elevation acute coronary syndrome (ACS), and the drug given with aspirin and eptifibatide improved patient outcomes, which were determined on the basis of better safety and efficacy results, as compared with UFH, though major bleeding was more frequent in the enoxaparin group (12). Based on these recent trials, it is widely recognized that LMW heparin is useful for thromboprophylaxis in patients with ACS as compared with UFH, however, the incidence of bleeding is greater.

#### 4.1.2. Effects of LMW heparin for treatment of thromboembolism

Venous thromboembolism (VTE) is a major cause of morbidity and mortality in hospitalized patients (13). Nevertheless, thromboprophylaxis use in medical patients has not been universally accepted or adopted, even though medical patients are at risk for VTE (14). It was recently suggested that thromboprophylaxis is needed for medical as well as hospitalized patients, because of that increased risk (15).

Thromboprophylaxis with UFH has been shown to be effective in reducing the incidence of deep-vein thrombosis (DVT) and overall mortality in medical patients, while that with LMW heparin is reported to be at least as effective as with UFH, with the advantage of fewer bleeding complications (16). In 2 randomized

clinical trials of LMW heparin [Prophylaxis in Medical Patients with Enoxaparin (MEDENOX) and Prospective Evaluation of Dalteparin Efficacy for Prevention of VTE in Immobilized Patients Trial (PREVENT)], thromboprophylaxis with enoxaparin (40 mg subcutaneously, once daily) or dalteparin (5000 IU once daily) was more effective than placebos and well tolerated by the medical patients (17, 18). In addition, in a subgroup analysis in the PREVENT trial, a fixed dose (5000 U/day) of dalteparin was effective and safe in preventing VTE in obese or elderly hospitalized medical patients (19). Based on those results, LMW heparin has become recognized as more useful for thromboprophylaxis as compared with UFH, and its widespread use is now recommended for both medical and hospitalized patients.

4.1.3. Effects of LMW heparin for treatment of acute ischemic stroke

Nadroparin (Fraxiparine®) was reported to be effective for improving the 6-month outcome of patients with ischemic stroke who were treated within 48 hours of onset of symptoms (20), though such efficacy was not seen in more recent trials. In a randomized, double-blind, double-dummy trial [Heparin in Acute Embolic Stroke Trial (HAEST)] with 499 patients suffering from acute ischemic stroke and arterial fibrillation, dalteparin (100 IU/kg subcutaneously twice a day) was not superior to aspirin (160 mg every day) for the prevention of recurrent stroke and secondary events during the first 14 days, and there were no significant differences in functional outcome or death after 14 days or 3 months (21). Further, the rate of bleeding was higher among patients given dalteparin as compared to those given aspirin.

In another randomized, double-blind, dose-finding multicenter trial [Therapy of Patients With Acute Stroke (TOPAS)] with 404 patients with acute ischemic stroke, a dosage increase of certoparin (Sandoparin®) up to 8000 U twice daily did not improve the functional outcome of patients (22). In addition, the highest dose of certoparin was associated with the highest rate of bleeding, with no differences in the rates of favorable outcomes noted among the tested dosages.

Since LMW heparin is associated with an increased risk of serious bleeding complications when used for prevention of acute ischemic stroke, strong evidence for its efficacy is needed in order to justify its use for urgent anticoagulation.

4.2. Heparinoid

Danaparoid sodium (Orgaran®) is a low molecular weight heparinoid that consists of sulfated glycosaminoglycans derived from porcine intestinal mucosa that contains 84% heparan sulphate, 12% dermatan sulphate, and 4% chondroitin sulphate. Danaparoid exerts a stronger catalytic effect on the inactivation of FXa than on the inactivation of thrombin and has relatively few effects on platelet aggregation. The anti-FXa activity has a half-life of 24.5 hours, with 40-50% explained by renal clearance, and is mediated by AT and not inactivated by endogenous heparin neutralizing factors (23).

In experimental models, the antithrombotic activity of danaparoid has been shown to be more persistent than that of UFH, while the hemorrhagic effects are less persistent. The drug has also been submitted to clinical studies for use in continued anticoagulant therapy in patients with heparin-induced thrombocytopenia (HIT), for prophylaxis and treatment of DVT, and for treatment of disseminated intravascular coagulation (DIC), with the results reviewed by Ibbotson (24).

4.2.1. Effects of danaparoid with prophylaxis for deep vein thrombosis

Factors that contribute to the development of DVT include stasis, hypercoagulability, and vessel wall injury, all of which are sequelae of major surgery and immobility (25). Several randomized trials have demonstrated that danaparoid is effective and safe for the prevention of postoperative VTE in patients undergoing general or orthopedic surgery. Further, randomized and comparative studies using patients undergoing hip surgery (hip fracture or hip replacement surgery) have shown that danaparoid significantly reduces the incidence of postoperative DVT as compared with aspirin (26), warfarin (27, 28), dextran 70 (29), heparin dihydroergotamine (30), and a placebo (31). In those reports, a total of 120 (14.0%) patients received danaparoid (750 U once or twice daily) among 859 patients who had evidence of DVT and underwent hip surgery, which was significantly fewer than those (29.8%) who received another drug or a placebo (56.5%), while there was no significant difference with enoxaparin (15.4%) or dalteparin (8.8%) (24).

In patients undergoing abdominal and thoracic surgery for removal of a malignancy, danaparoid reduced the incidence of postoperative DVT as compared with a placebo, however, showed no significant difference when compared with UFH (32, 33). In short, only 8.5% of the patients who received danaparoid (500-1000 U subcutaneously twice daily) developed DVT, which was significantly fewer than those (64%) who received a placebo, though not significantly different from those (11.2%) who received UFH. However, the development of DVT was slightly higher (36%) in the patients who received the smaller dose (500 U) of danaparoid. On the other hand, the drug was reported to be effective for prophylaxis for DVT following acute ischemic stroke, while it has been also been suggested to be useful without prophylaxis, as DVT develops in 28% to 75% of stroke patients (34). When used with prophylaxis for DVT following acute ischemic stroke, danaparoid was significantly superior to UFH without a significant difference in side effects (32, 33). Further, in patients with DIC, 61.9% of those patients who received danaparoid experienced either a disappearance or reduction of symptoms, whereas 62% of those who received UFH showed either no change or an aggravation of symptoms (24).

4.2.2. Effects of danaparoid for treatment of acute ischemic stroke

Danaparoid has been expected to be effective in preventing DVT in stroke patients similar to LMW heparin, however, there are few reports of its efficacy when used as therapy for acute stroke. In a randomized, double-blind, placebo-controlled, multicenter trial [Trials of ORG-10172 in Acute Stroke Treatment (TOAST) (35)] with 1281 patients with acute stroke, danaparoid

was given initially as a bolus within 24 hours of the stroke followed by continuous infusion for 7 days at a dose adjusted to maintain the anti-FXa activity at 0.6-0.8 U/ml. However, it was not found to be associated with an improvement in favorable outcome after 3 months, though there was an apparent positive response to treatment at 7 days. Those authors noted that administration as late as 24 hours after onset might improve the outcome of patients whose strokes are secondary to large-artery atherosclerosis, and also found that emergency administration of danaparoid was associated with major bleeding and an increased risk of intracranial hemorrhage, especially among patients who experienced a major stroke.

4.2.3. Effects of danaparoid for treatment of heparin-induced thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is an immune-mediated syndrome that develops in 1% to 3% of patients receiving UFH (36). HIT is usually manifested within 5-10 days following the start of heparin treatment and is associated with antibodies, usually of the IgG class, directed against heparin-platelet factor 4 complexes as the major antigen (37). From 30% to 75% of patients with HIT develop thrombosis, with venous events about four times as common as arterial events. However, cross-reactivity between danaparoid and the heparin-associated antibody from the plasma of patients with HIT is less than 10% (38). Several trials with danaparoid have been performed in patients with both thromboembolism and HIT.

In a small prospective randomized trial, danaparoid plus warfarin was superior to dextran 70 plus warfarin in HIT patients with severe thromboembolic complications (39). In an open-labeled trial with 42 patients with recent thrombosis and a clinical diagnosis of HIT, for whom danaparoid was given at a dose of 2400 U as a bolus injection followed by 400 U/hour for 2 hours, 300 U/hour for 2 hours, then 200 U/hour for 5 days, there was complete clinical recovery from thromboembolic events in 56% of the patients, as compared to 14% after dextran 70 given at 1000 ml on day 1 and then at 500 ml on days 2 through 5 (odds ratio: 10.5, IC95%: 1.2-16.7, p=0.02). Overall clinical effectiveness of danaparoid was rated as high or moderate in 88% of the patients, as compared with 47% for dextran 70 (p=0.01) (39). Further, a retrospective analysis compared the use of lepirudin, a recombinant thrombin inhibitor, and danaparoid for anticoagulant therapy in patients with HIT (40). The cumulative risk of combined end point (new thromboembolic complications, amputations and/or death) was higher in HIT patients without thromboembolic complications at the baseline treated with prophylactic dose of danaparoid as compared to lepirudin (p=0.02). Whereas HIT patients with thromboembolic complications at baseline treated with the therapeutic dose had a similar outcome in both treatment groups. The therapeutic doses were at least twice as great as the prophylactic doses. Major bleeding occurred in 2.5% of the danaparoid treated patients as compared to 10.4% of the lepirudin treated patients until day 42 (p=0.009). In another trial with 45 patients with HIT type II, danaparoid was given intravenously at 2.6 IU/kg/hour (therapeutic use), which led to a fast normalization of platelet counts, while that given subcutaneously at 10 IU/kg with a vitamin K-antagonist (as a prophylactic) was effective for the prevention of thromboembolic complications (41).

Therefore, danaparoid may be effective for patients with HIT. However, it was recently reported that 5 cases of HIT treated with danaparoid developed both thrombocytopenia and new thromboembolic complications, suggesting that a low or intermediate dosage may be inadequate for treatment of HIT (42).

4.3. Synthetic Pentasaccharide

A synthetic pentasaccharide, such as fondaparinux and SANORG-34006, is a selective and indirect inhibitor to FXa, with fondaparinux currently used for the prevention of and therapy for thromboembolism.

Fondaparinux (Arixtra®) is a novel synthetic pentasaccharide with a MW of 1,728 daltons that binds with a high affinity to the heparin binding site of AT (Kd = 48 nM) and is the first FXa inhibitor approved for use in thromboprophylaxis following orthopedic surgery. The half-life of the drug is from 13 to 21 hours, allowing a once daily administration (44). Unlike UFH and LMW-heparin, pentasaccharide does not interact with platelets and prothrombin, making its anticoagulant effects more selective and predictable. At recommended doses, it neither activates fibrinolysis nor prolongs bleeding time. Its antithrombotic effects in clinical trials have been well reviewed by Leone (45) and Bauersachs (46).

SANORG-34006 (idraparinux) also has a high affinity to human AT (Kd = 1.4 nM), and is a potent and selective catalyst of the inhibitory effect of AT on FXa (47). Idraparinux has a half-life of approximately 4 days, making it suitable for a once a week subcutaneous injection (48). After both intravenous and subcutaneous administrations to rabbits, the drug displayed long-lasting antithrombotic activity by virtue of its potentiation of the anti-factor Xa activity of AT, and strongly inhibited thrombin formation in experimental rat models of thromboplastin-induced venous thrombosis that received a subcutaneous administration ($ED_{50}$ = 40.0 nM/kg) and in rabbits that received an intravenous administration ($ED_{50}$ = 105.0 nM/kg) (47). Idraparinux was also reported to enhance recombinant tissue-plasminogen activator (rtPA)-induced thrombolysis and inhibit accretion of $^{125}$I-fibrinogen into a preformed thrombus in rabbit jugular veins, suggesting that its concomitant use during rtPA therapy might be helpful in facilitating thrombolysis. Further, idraparinux did not enhance bleeding in a rabbit ear incision model at a dose that equaled 10 times the antithrombotic $ED_{50}$ in that species and exhibited a favorable therapeutic index (47).

SANORG-123781A is the first example of a totally synthetic hexadecasaccharide that exhibits the AT-mediated inhibitory activities of both FXa and thrombin with no binding to PF-4 (49). The drug also shows a high affinity for human AT (Kd = 58.22 nM), and is a potent catalyst of its inhibitory effect with regard to FXa ($IC_{50}$ = 77 ng/ml) and thrombin ($IC_{50}$ = 4.0 ng/ml). Although SANORG-123781A retains the full antithrombotic properties of UFH, this agent does not compete for $^{3}$H-heparin binding to platelet factor 4 (PF4) or activate platelets in the presence of plasma from patients with HIT (50). Following intravenous and subcutaneous administration to animal models,

Table 2. Phase III trials of prophylaxis for venous thromboembolism with fondaparinux

| Study (Reference) | Methods (no. of patients) | Indication regimen | Fondaparinux | Comparator | Efficacy (fondaparinux vs. comparator) | Safety (fondaparinux vs. comparator) |
|---|---|---|---|---|---|---|
| PENTHIFRA (105) | Superiority trial (1,711) | Major orthopedic surgery (hip fracture) | 2.5 mg once daily starting postoperatively | Enoxaparin 40 mg once daily starting preoperatively | VTE 8.3% vs. 19.1%, RRR=56% P<0.001 | Clinical relevant major bleeding 0.2% vs. 0.3% |
| PENTAMAKS (106) | Superiority trial (1,049) | Major Orthopedic surgery (major knee surgery) | 2.5 mg once daily starting postoperatively | Enoxaparin 30 mg twice daily starting postoperatively | VTE 12.5% vs. 27.8%, RRR=55% P<0.001 | Clinical relevant major bleeding 0.4% vs. 0.2% |
| EPHESUS (107) | Superiority trial (2,309) | Major Orthopedic surgery (total hip replacement) | 2.5 mg once daily starting postoperatively | Enoxaparin 40 mg once daily starting preoperatively | VTE 4.1% vs 9.2%, RRR=56% P<0.001 | Clinical relevant major bleeding 0.4% vs.0.3% |
| PENTATHLON 2002 (108) | Superiority trial (1,175) | Major orthopedic surgery (total hip replacement) | 2.5 mg once daily starting postoperatively | Enoxaparin 30 mg twice daily starting postoperatively | VTE 6.1% vs. 8.3%, RRR=26% | Clinical relevant major bleeding 0.2% vs. 0.3% |
| PEGASUS (54) | Superiority trial (2,927) | High-risk abdominal surgery | 2.5 mg once daily starting postoperatively for 5-9 days | Dalteparin 2500 IU 2 hrs preoperatively, then 12 hrs post-operatively and 5000 IU once daily for 5-9 days | VTE 4.6% vs. 6.1%, RRR=25% P=0.14 | Major bleeding 3.4% vs. 2.4% |
| PENTHIFRA Plus (61) | Superiority trial (656) | Major orthopedic surgery (hip fracture) | 2.5 mg once daily starting postoperative | Fondaparinux 2.5 mg once daily for 7 days, followed by placebo for 25-31 days | VTE 1.4% vs. 35%, RRR=96% P<0.001 | Clinical relevant Major bleeding 0.6% vs. 0.6% |
| ARTEMIS (53) | Superiority trial (847) | Acutely ill medical patients | 2.5 mg once daily for 6-14 days | placebo | VTE 5.6% vs. 10.5% RRR=47%, P<0.05 | Major bleeding 0.2% vs. 0.2% |

VTE: venous thromboembolism, RRR: relative risk reduction

SANORG-123781A displayed prolonged anti-FXa activity and anti-thrombin activity *ex vivo*, and the intravenous administration strongly inhibited thrombin formation in a rat experimental model of thromboplastin-induced venous thrombosis ($ED_{50}$ = 18 $\mu$g/kg, vs. 77 $\mu$g/kg for UFH). However, bleeding in various experimental models occurred at higher doses, though it has been reported to exhibit a highly favorable antithrombotic/bleeding ratio (50).

4.3.1. Effects of fondaparinux for the prevention of venous thromboembolism

Fondaparinux has been approved by the FDA at a daily dosage of 2.5 mg for the prevention of VTE in patients who have undergone major orthopedic surgery. Trials for the potential use of this agent for the prevention of VTE were conducted in both surgical and medical settings, as well as for the treatment of established VTE. In a phase II dose-ranging study (PENTATHLON trial) with patients undergoing surgery for elective total hip surgery, the thromboprophylactic dosage regimen of fondaparinux for orthopedic surgery cases was determined (51). When fondaparinux was administered subcutaneously once daily at 5 different doses (0.75, 1.5, 3.0, 6.0, and 8.0 mg), or LMW heparin (enoxaparin) was administered at a dose of 30 mg for comparisons, fondaparinux showed a statistically significant dose-dependent efficacy for the incidence of VTE as well as safety, as the rate of VTE was significantly lower in the fondaparinux 3.0 mg group (1.7%) than in the enoxaparin group (9.4%) (p = 0.01), while the incidence of major bleeding was comparable (fondaparinux; 4.5%, enoxaparin; 3.5%). Those results and statistical calculations suggested a dose of 2.5 mg for the phase III trials. In randomized, double-blind international phase III trials in patients undergoing surgery for hip fracture, elective hip replacement, and major knee surgery, fondaparinux administered at a subcutaneous dose of 2.5 mg (once daily) postoperatively, reduced the overall incidence of VTE up to day 11 by 55.2% (p<0.001 vs. enoxaparin) (52) (Table 2). In another randomized double-blind trial, 4 weeks of prophylaxis with fondaparinux after hip fracture surgery reduced the risk of VTE by 96% as compared to 1 week of the prophylaxis, and it was well tolerated (53). Further, fondaparinux (2.5 mg once daily starting postoperatively) given to high-risk abdominal surgery patients reduced the incidence of VTE to 4.6% from 6.1% with dalteparin (2500 IU, 2 hours preoperatively, then 12 hours postoperatively and 5000 IU once daily for 5-6 days) (54). In acutely ill medical patients who received 2.5 mg of fondaparinux (once daily for 6-14 days), the incidence of VTE was smaller (5.6%) than that (10.5%) with a placebo (53).

Fondaparinux has been approved for the prevention of VTE following major orthopedic surgery, and is at least as effective and safe as current reference drugs for treatment of VTE, though additional trials should be conducted, because the timing of the first dose of fondaparinux was different in previous trials. Recent studies of fondaparinux for the treatment of VTE include 2 different phase III, large-scale, randomized clinical trials. Both the efficacy and safety of once daily

Table 3. Phase III trials for treatment of venous thromboembolism with fondaparinux

| Study (Reference) | Methods (Patients No.) | Indication regimen | Fondaparinux | Comparator | Efficacy (fondaparinux vs. comparator) | Safety fondaparinux vs. comparator) |
|---|---|---|---|---|---|---|
| MATISSE-PE (56) | Superiority trial (2,205) | Symptomatic pulmonary embolism | 7.5 mg once daily (5 mg for body weight <50 kg or 10 mg for body weight >100 kg) | adjusted-dose continuous intravenous UFH | VTE 3.8% vs. 5%, RRR=24% | Major bleeding 2.0% vs. 2.4% |
| MATISSE-DVT (55) | Superiority trial (2,213) | Symptomatic deep-vein thrombosis | 7.5 mg once daily (5 mg for body weight <50 kg or 10 mg for body weight >100 kg) | Enoxaparin 1 mg/kg twice daily | VTE 3.9% vs. 4.1% | Major bleeding 1.1% vs. 1.2% |

VTE: venous thromboembolism, RRR: relative risk reduction, UFH: unfractionated heparin

fondaparinux (2.5 mg) were at least as good as those of enoxaparin (1 mg/body kg twice daily) for the treatment of VTE (MATISSE-DVT) (55) and of UFH (adjusted-dose continuous) for the treatment of pulmonary embolism (MATISSE-PE) (56) (Table 3). A once-daily, subcutaneous administration of fondaparinux without monitoring is considered to be at least as effective and safe as adjusted-dose UFH as initial treatment for hemodynamically stable patients with pulmonary embolism (PE). An advantage of fondaparinux is that the regular dose does not require monitoring of anticoagulant activity or adjustments.

Although this pentasaccharide has been reported to have few hemorrhagic effects, recombinant FVIIa is useful to reverse the anticoagulant effect of fondaparinux in case of serious bleeding complications or when surgery is needed during treatment with the drug (57).

4.3.2. Effects of fondaparinux for prevention and treatment of coronary events

Anticoagulants have a prominent position among the various types of therapy used for the management of patients with ACS. Fondaparinux has been compared with heparin for treatment of patients following a coronary event. The PENTALYSE study was a dose-ranging study that compared fondaparinux with UFH in 333 patients treated with aspirin and rt-PA (alteplase) (58). Fondaparinux (daily dose of 4-6 mg, 6-10 mg, or 10-12 mg for 5-7 days) was well tolerated and as effective as UFH in maintaining arterial potency following thrombolysis in patients with myocardial infarction, while the incidence of major bleeding was 7.1% in both groups.In a recent randomized trial [Arixtra Study in Percutaneous Coronary Intervention: A Randomized Evaluation (ASPIRE) Pilot Trial] with 350 patients undergoing elective or urgent percutaneous coronary intervention (PCI), the composite efficacy outcome of all-cause mortality, myocardial infarction, urgent vascularization, or need of bailout glycoprotein (GP) IIb/IIIa antagonist, was 6.0% in both the UFH and 6.0% fondaparinux groups, with no significant difference in efficacy seen between the fondaparinux doses (2.5 mg or 5.0 mg) as compared with UFH. Further, the incidence of total bleeding was 7.7% in the UFH group, as compared to 3.4% in the 2.5 mg fondaparinux group and 9.6% in the 5.0 mg fondaparinux group (p=0.06, vs.2.5 mg fondaparinux group) (59). These data suggested further evaluation of fondaparinux in a large number of patients in the same setting. Two international, randomized, double-blind, controlled, parallel group studies, the OASIS-5 trial, which included patients with unstable angina and non-ST segment elevation myocardial infarction, and the OASIS-6 trial, which included patients with acute ST elevation myocardial infarction, are presently being conducted. A large proportion of those patients are undergoing PCI.

Fondaparinux was also investigated in an open pilot study as an adjunct to percutaneous transluminal coronary angioplasty (PTCA), which is known to be a clinical setting that promotes arterial thrombosis. PTCA was performed in 61 patients with stable or unstable angina for more 7 days, or acute myocardial infarction with more than 70% stenosis. A 5-minute intravenous infusion of 12 mg of fondaparinux given prior to angioplasty along with 500 mg of intravenous aspirin was found to be effective and safe. The abrupt vessel closure rate was 3.3%, with an efficacy comparable to that seen in historical trials of heparin (4.2% to 8.3%), and no major bleeding occurred. Thus, fondaparinux seems to be as effective and safe as heparin in patients with coronary artery diseases undergoing PTCA (60).

4.3.3. Effects of fondaparinux for heparin-induced thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is a complication of heparin therapy caused by antibodies against a complex of PF4 and heparin. No severe thrombocytopenia was reported during prolonged administrations of 2.5 mg of fondaparinux for up to 4 weeks in the PENTHIFRA plus study (61). Overall, no episode of HIT was reported in either the phase II or phase III program. Previous reports have found that fondaparinux shows no cross-reactivity with antibodies to heparin-PF4 complexes (62, 63). In a recent serological study to determine the cross-reactivity of HIT sera with fondaparinux, the drug was significantly less reactive (3.3%) than UFH (79.8%) in the presence of HIT sera ($p<0.001$) and found not to induce the binding of HIT-associated (anti-PAC-1 and anti-CD62) antibodies to platelets with HIT sera, using a flow cytometry technique (64). Thus, fondaparinux may be useful for prophylaxis and treatment of thrombosis in patients with a history of HIT, though more clinical experience is required.

## 5. SYNTHETIC SELECTIVE DIRECT INHIBITORS OF FACTOR Xa

A large number of synthetic, selective, and direct inhibitors of FXa are currently being developed worldwide, as FXa is a key enzyme in the coagulation

Table 4. Synthetic selective direct FXa inhibitors

| Inhibitor | Ki for FXa | Ki for thrombin | Peak plasma level (after dosing) | Plasma half-life |
|---|---|---|---|---|
| DX-9065a (Daiichi Seiyaku) | 41 nM | > 2,000 $\mu$M | 1 hr (orally) | 2.3 hrs (orally)<br>3(α), 3.2(β), 83(γ) hrs (intravenously) |
| YM-466 (Yamanouchi Pharmaceutical) | 1.3 nM | > 100 $\mu$M | 0.5-1 hrs (orally)[1] | 0.5-1.5 hrs (orally)[1]<br>0.12 (α), 1.6 (β) hrs (intravenously)[1] |
| JTV-803 (Japan Tobacco) | 19 nM | > 100 $\mu$M | 2 hrs (orally)[2] | 3.6 hrs (orally)[2]<br>0.2(α), 2.1(β), 11.1(γ) hrs (intravenously)[2] |
| KFA-1411 (Kissei Pharmaceutical) | 1.73 nM | 2.6 $\mu$M | ------ | ------ |
| BAY-59-7939 (Bayer HealthCare AG) | 0.4 nM | > 20 $\mu$M | 0.5 hrs (orally) | 3-4 hrs (orally) |
| DPC-423 (Bristol-Myers Squibb) | 0.15 nM | 6.0 $\mu$M | 1-6 hrs (orally) | 27-35 hrs (orally)<br>7.5 hrs (intravenously)[3] |
| DPC-906 [razaxaban] (Bristol-Myers Squibb) | 0.19 nM | ----- | ----- | ----- |
| RPR-209685 (Aventis) | 1.1 nM | ----- | ----- | 0.87 hrs (orally)[3] |

[1]: data in squirrel monkeys, [2]: data in cynomolgus monkeys, [3]: data in dogs

cascade and an essential component of prothrombinase complex. Further, direct inhibition of FXa is considered to be effective for both venous and arterial thrombosis, and a direct inhibitor is expected to provide an improved side effect profile. Although these advantages are known, effective synthetic direct inhibitors are still in the process of development. *In vitro* and *in vivo* studies of direct inhibitors have been presented, however, there are few clinical reports of their use for prophylaxis or treatment of thrombosis. Most of these inhibitors have been developed in anticipation of orally active inhibitors of FXa and those presently available are shown in Table 4.

5.1. Antithrombotic effects of direct inhibitors

5.1.1. DX-9065a

DX9065a (molecular weight [MW]: 571.07) (Figure 2-A) is a highly selective and competitive inhibitor of FXa, as its estimated dissociation content (Ki) for FXa is reported to be 41 nM, while that for thrombin is > 2000 $\mu$M (65). Although DX-9065a inhibits both FXa and prothrombinase complex with similar Ki values in either the presence or absence of prothrombin, it also acts as a non-competitive inhibitor (Ki=~26 nM) of the proteolytic activity of prothrombinase during the process of prothrombin activation (66). DX-9065a has a three-compartment distribution (α, β, and γ half-lives of 0.23, 2.9, and 89.9 hours, respectively) and is cleared renally when intravenously administered at therapeutic doses (67). In contrast, the maximum plasma concentration of DX-9065a orally administered at 10 mg to healthy Japanese volunteers was 6.2 ng/ml at 1 hour after administration (68).

There are a number of reports of the strong inhibitory effect toward FXa by DX-9065a. Using a chromogenic assay with purified coagulation factors, 73.9% of thrombin generation was suppressed by 0.2 $\mu$M of DX-9065a (114.2 ng/ml), which was within the therapeutic dosage range expected in humans. In contrast, argatroban, a selective thrombin inhibitor, was less inhibitive (36.0%), as shown in Figure 3 (69). In addition, the inhibitory effect of DX-9065a (0.2 $\mu$g/ml) with LMW heparin (enoxaparin; 0.5 U/ml) was 2.4-fold greater than that of DX-9065a or LMW-heparin alone when measuring the inhibition of FXa activity in an *in vitro* study using a chromogenic assay with pooled plasma from healthy donors. These results suggest that a systemic state of moderate-to-high intensity anticoagulation should be anticipated in clinical settings where LMW heparin concentrations in excess of 0.5 U/ml coexist with DX-9065a levels of 0.2 $\mu$g/ml or greater (70), and further suggest that the binding site of DX-9065a to FXa is different from that of heparin (71). In an open-label crossover study with 6 healthy individuals, administration of DX-9065a alone or combined with aspirin significantly inhibited thrombus formation at high shear rate by 30% to 40% and by approximately 20% under low shear rate conditions, while enoxaparin did not have a significant effect (72). Thus, the combination of direct inhibitors and other anticoagulant agents is expected to be effective for thromboprophylaxis as compared with an anticoagulant alone, however, additional clinical studies including those with a focus on the hemorrhagic side effects are necessary prior to clinical use.

There are also a number of *in vitro* studies and disease model studies that show the efficacy of DX-9065a toward FXa inhibition. The inhibitory effect of DX-9065a appears to also affect FXa present on the surface of microparticles, which can display a pro-thrombotic effect. Heault reported that DX-9065a was a potent antithrombotic compound when thrombosis was induced by platelet-derived microparticles in an *in vivo* study with a modified AV shunt rat model (73). DX-9065a, but not heparin, reduced the mortality at 6 hours after the initiation of venous thrombi in mice with experimental venous thrombosis and PE (74). Moreover, when DX-9065a was administered to PAI-1 knockout mice with PE, the mice survived well without marked bleeding. Coagulation caused by FXa may play a role in the amplification of PE, and PAI-1 may play a key role in embolism development. Therefore, a dual inhibition of

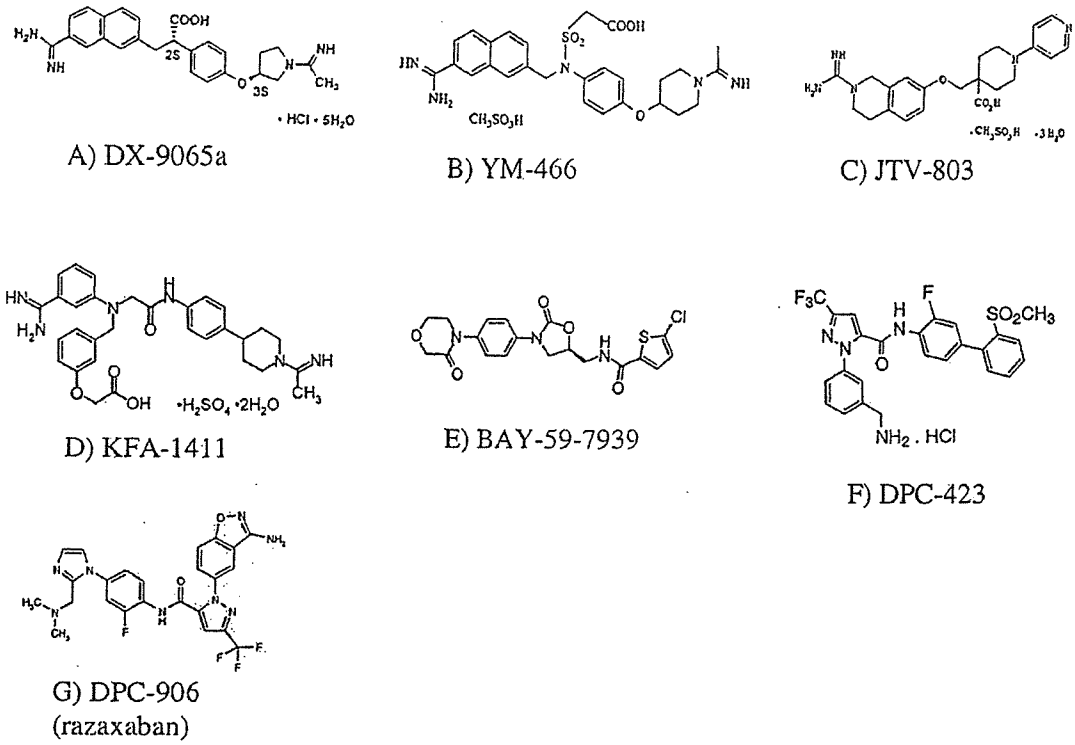


Figure 2. Structural formulae of synthetic direct FXa inhibitors.

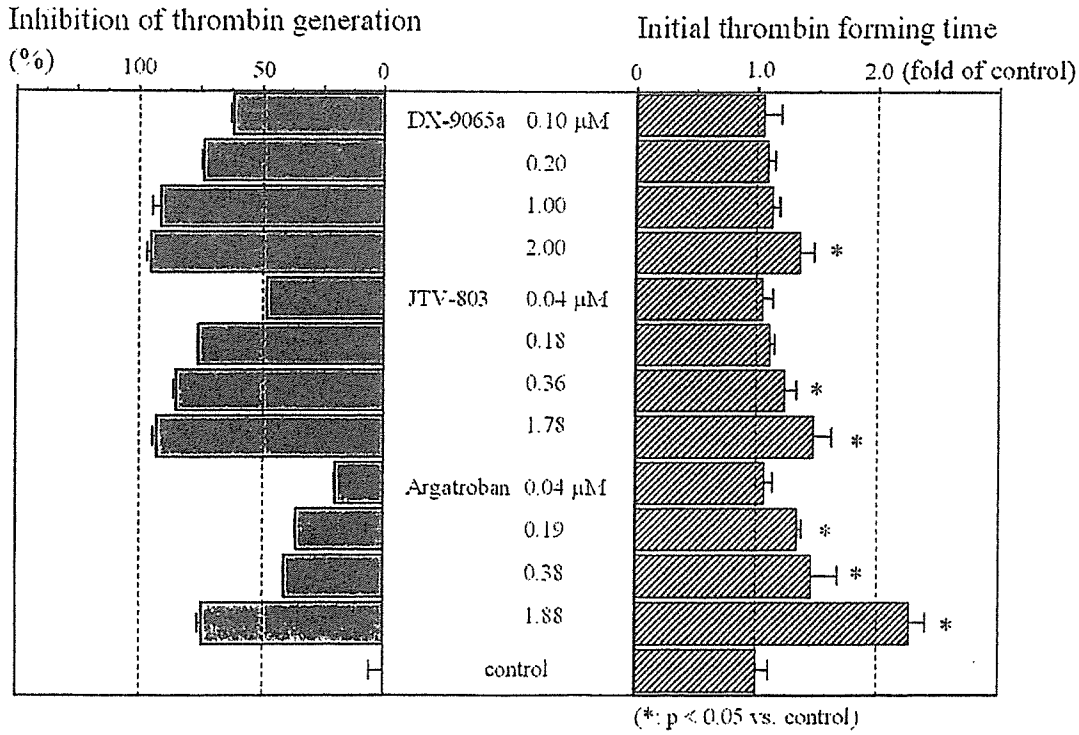


Figure 3. Influence of antithrombotic agents on thrombin generation and initial thrombin forming time. Direct inhibitors to FXa (DX-9065a and JTV-803) did not affect the initial thrombin formation, which is considered important for platelet aggregation in hemostasis, whereas thrombin generation was strongly suppressed by the addition of the inhibitors. In contrast, argatroban inhibited both thrombin generation and initial thrombin formation.

coagulation for FXa and PAI-1 significantly improved the rate of mortality in acute PE (74). Also, DX-9065a has been found to exert an effective protection against experimental tumor-induced DIC in rats, suggesting that this agent can improve the hypercoagulable state induced by progress of a solid tumor (75).

5.1.2. YM-466

YM-466 (Figure 2-B) is also a potent, specific, and orally active inhibitor of FXa that has a high affinity for FXa (Ki = 1.3 nM), though it does not affect thrombin (Ki > 100 $\mu$M) (76). YM-466 inhibits FXa in prothrombinase complex with an $IC_{50}$ value of 7.7 nM and platelet aggregation induced by various agonists ($IC_{50}$ = 3 to 23 $\mu$M), probably by inhibiting the binding of fibrinogen to GP IIb/IIIa on platelet surfaces (76).

In an electrically-induced carotid artery thrombosis rat model, YM-466 intravenously administered at 1 mg/kg/hour before electrical stimulation improved patency status, and prolonged the time to occlusive thrombus formation and duration of patency, as compared with UFH, dalteparin, and argatroban (77). In the same study, YM-466 orally administrated at 30 mg/kg before electrical stimulation significantly reduced the incidence of occlusion and improved carotid artery patency, which was in contrast to ticlopidine, cilostazol, aspirin, beraprost, ethyl icosapentate, and warfarin (77). Thus, YM-466, which can be given orally and intravenously, was shown to be an effective antithrombotic agent for the treatment and prevention of arterial thrombosis. In addition, the absorption, distribution, metabolism, and extension of YM-466 were investigated in rats following a single oral administration. The plasma concentration of orally given YM-466 reached a maximum within 30 minutes, and non-metabolic YM-466 was rapidly distributed to the livers and kidneys, with 76.1% and 25.2% of the administered amount excreted in feces and urine, respectively (78).

A combination of YK-466 at 10 mg/kg with YM-128, a GPIIb/IIIa antagonist, at 3 mg/kg was reported to inhibit thrombotic occlusion and neointima formation in mice, without affecting platelet aggregation, suggesting that the concomitant inhibition of FXa and GPIIb/IIIa may provide a safe and effective therapeutic regimen for treatment of coronary angioplasty (79). However, the problem of hemorrhagic side effects remains in clinical use, because platelet function is also inhibited by YK-466 alone (76).

5.1.3. JTV-803

JTV-803 (Figure 2-C) also shows a competitive inhibitory effect toward human FXa. The Ki of JTV-803 (MW: 559.64) for FXa was found to be 19 nM, with an effective dose ranging from approximately 0.1 to 0.5 $\mu$M (80). Using a chromogenic assay with purified coagulation factors, 75.7% of thrombin generation was significantly suppressed by the addition of JTV-803 (0.18 $\mu$M), which was within the therapeutic dosage expected in humans, as compared with argatroban (36.0%) (Table 5) (69). JTV-803 has also been shown to inhibit thrombus formation in an AV shunt model (81), and was demonstrated to be effective for treating both liposaccharide-induced and tissue factor-induced DIC in rat models (82).

5.1.4. KFA-1411

KFA-1411 (Figure 2-D) also selectively inhibits FXa with a Ki value of 1.73 nM (Ki value for thrombin, 26,000 nM) without inhibition of platelet aggregation at a concentration shown to have an anticoagulant action. The anticoagulant action of KFA-1411 in human plasma is nearly equal to that of the selective thrombin inhibitor argatroban (83).

5.1.5. BAY-59-7939

BAY-59-7939 (Figure 2-E) is an oral, direct inhibitor to FXa, with a molecular weight of 435.89 daltons. This agent inhibits human factor Xa (Ki = 0.4 nM) with a greater than 1000-fold selectivity as compared to other serine proteases. BAY-59-7939 also inhibits endogenous factor Xa in more potent manner in human plasma ($IC_{50}$ = 21 nM) than rat plasma ($IC_{50}$ = 290 nM) (84).

The effects of BAY-59-7939 administered intravenously and orally in both arterial and venous thrombosis models were investigated. When 0.1 mg/kg was intravenously administered, venous thrombosis was reduced in a dose dependent manner in a venous stasis rat model, while 5.0 mg/kg administered orally reduced arterial thrombus formation in an AV shunt in rat model. Further, BAY-59-7939 was reported to slightly inhibit FXa (32% at $ED_{50}$) and reduce thrombus formation in a venous model, though a stronger inhibition of FXa was required to affect arterial thrombosis in rat and rabbit models (74% and 92%, respectively, at $ED_{50}$) (84). In a multiple dose escalation study with BAY-59-7939, maximal FXa inhibition of 70% was achieved at a steady state with the highest dose (30 mg, twice daily) (85). Further, the agent did not directly affect platelet aggregation *in vitro* (86), or increase bleeding time in a clinical study with healthy donors (85, 87).

5.1.6. DPC-423

DPC-423 (Figure 2-F) is another synthetic, competitive, and selective inhibitor of FXa that inhibits human FXa with Ki value of 0.15 nM (Ki: 6000 nM for human thrombin) (88). When given to dogs, DPC-423 produced a pharmacokinetic profile with an oral bioavailability of 57%, a plasma clearance of 0.24 l/kg/hour, and half-life of 7.5 hours (89). In a preliminary study with healthy donors, DPC-423 was well tolerated and orally bioavailable, with a plasma half-life of 27-35 hours. Further, the effective plasma concentration ($EC_{50}$) value was 137 nM in an electric current-induced arterial thrombosis rabbit model, and the antithrombotic effect of DPC-423 was significantly correlated with its *ex vivo* anti-FXa activity (r=0.89), determined using a chromogenic assay (88).

5.1.7. DPC-906 (razaxaban)

DPC-906 (razaxaban, MW: 528.5) (Figure 2-G) is a potent and selective inhibitor of FXa that inhibits human FXa with a Ki value of 0.19 nM (540 nM for human thrombin). This inhibitor has been shown to be orally bioavailable in rats, dogs, and humans, as well as highly constrained to fit into the FXa active site in a complementary manner, while it showed a doubling of aPTT and PT at 6.1 and 2.1 $\mu$M, respectively (89).

Another study found that DPC-906 was efficacious in a rabbit AV shunt thrombosis model with an $IC_{50}$ value of 340 nM and electrically induced carotid artery thrombosis model rabbits with an $EC_{50}$ value of 379 nM, when given as an intravenous infusion 1 hour before the initiation of thrombosis (90).

5.2. Clinical trials of direct inhibitors

Over the last decade, significant advances in the development of anticoagulants including synthetic direct FXa inhibitors have been made for use with pharmacotherapy for ACS. Although aspirin and UFH are the primary agents given to patients with ACS, additional approaches are considered necessary to combat the intense inflammatory and thrombotic cascades invoked by the disease. Indeed, LMW-heparin and fondaparinux have been reported to have greater anti-FXa activities than UFH, and are considered superior for treating patients with ACS (92, 93).

In a double-blind trial [Xa Neutralization for Atherosclerotic Disease Understanding (XaNADU) IB study] of 73 patients with stable coronary artery disease, infusion of DX-9065a for 72 hours (produced concentration, 14-324 ng/ml) was well tolerated without major bleeding and serious adverse events were not reported during the infusion period. Further, it was noted that the drug concentration in plasma correlated strongly with anti-FXa activity (r=0.97) in that study (67). In another report of an initial experience with FXa inhibition in PCI (XaNADU- PCI pilot), direct FXa inhibition was reported to be a novel and potentially promising approach to anticoagulation during PCI (94). In a randomized, open-label study with 175 patients, elective PCI was feasible using a direct FXa inhibitor as an anticoagulant and predictable plasma drug levels were rapidly obtained with a double-bolus infusion of DX-9065a, 15 minutes after which the concentration was greater than 176 ng/ml (94). Additional studies are necessary to establish the efficacy of DX-9065a in patients undergoing PCI. However, based on these previous studies, in patients with stable coronary artery disease or undergoing PCI, the effective dose of DX-9065a for clinical trials has been set in a range of 100 to 200 ng/ml of plasma concentration, which is expected to be maintained by measuring anti-FXa activity and whole-blood INR (67, 72, 94).

In a phase II trial of DX-9065a in 402 patients with non-ST-elevation ACS (XaNADU-ACS trial) (95), there was no-significant tendency toward a reduction in ischemic events and bleeding with DX-9065a as compared with UFH. The primary efficacy endpoint (death, myocardial infarction, urgent revascularization, or ischemia on continuous ST-segment monitoring) occurred in 33.6%, 34.3%, and 31.3% of patients assigned to the UFH (bolus at 70 U/kg, then 15 U/kg/hour infusion up to 72 hours), low-dose DX-9065a (bolus at 0.025 mg/kg, then 3 hour loading infusion at 0.04 mg/kg, followed by a maintenance infusion at 0.012 mg/kg/hour up to 72 hours), and high-dose DX-9065a (2-fold volume of low-dose group) groups, respectively, with major bleeding occurring in 3.3%, 0.8%, and 0.9%, respectively. All of the patients also received aspirin, 325 mg initially and 81-325 mg daily. However, higher concentrations of DX-9065a were associated with a lower likelihood of ischemic events (P = 0.03) and a non-significant tendency toward a high likelihood of major bleeding (P = 0.32) (95). These results suggest that additional investigations are needed in patients with ACS.

DPC-906 (razaxaban) has also been studied in a phase II randomized, double-blind, multicenter trial, in which several dosages were compared to enoxaparin in knee replacement patients. In that trial, 656 patients undergoing elective primary total knee replacement surgery were randomly assigned to receive razaxaban twice daily at doses of 25 mg, 50 mg, 75 mg, or 100 mg, which were started 8 hours after surgery and given for 10 days, or subcutaneous enoxaparin twice daily at 30 mg that was started 12 to 24 hours after surgery and given for 10 days. The 3 highest doses of razaxaban were each associated with an increased rate of bleeding as compared to enoxaparin, however, patients on the lowest dose had a similar incidence of major bleeding compared to those on enoxaparin (0.7% vs.0.0%) and a lower rate of VTE (8.6% vs.15.9%). It has been concluded that razaxaban at a dose of 25 mg has a good potential for increased efficacy and similar safety as compared to standard treatment protocols (96).

4.3. Influences of direct inhibitors on laboratory tests and monitoring

During therapy with anticoagulants for thrombotic diseases, monitoring is important for maintaining the correct therapeutic dose. Clinically, heparin is typically monitored using either activated partial thromboplastin time (aPTT) or activated clotting time, while warfarin is usually monitored using prothrombin time (PT) or PT-INR. Although a number of studies have been performed, monitoring for DX-9065a is still not well established.

In an *in vitro* thrombin generation assay with purified human coagulation factors, DX-9065a at concentrations of 0.5 $\mu$M (285 ng/ml) or less had little or no significant effect on aPTT or PT (69). In contrast, in an open-label, crossover study with 6 healthy individuals, administration of DX-9065a to gain a plasma concentration in the range of 20–80 ng/ml induced a small but significant increase in both aPTT and PT (72). These results suggest that aPTT and PT levels may not reflect the antithrombotic activity of DX-9065a.In a clinical trial with patients with stable coronary disease, a predictable plasma drug level was shown to be correlated with anti-FXa activity following the infusion of DX-9065a (r=0.97), but less so with aPTT (r=0.56) (67). Further, in experiments with BAY-59-7939 in rat and rabbit AV shunt models, PT was increased by 1.2-fold and 3.2-fold, respectively, at the effective dose for 50% thrombus reduction, while plasma concentrations were 14-fold lower in the rabbits as compared with the rats (0.07 and 1.0 $\mu$M, respectively). In contrast, the levels of inhibition of FXa activity for a 50% reduction of thrombus were 74% and 92% in those rat and rabbit AV shunt models, respectively. These results suggest that antithrombotic efficacy can be predicted more precisely by the anti-FXa activity of the inhibitor in plasma than by PT (84). Monitoring is important during antithrombotic therapy with a direct FXa inhibitor to determine its efficacy and safety, though the effective dose of the inhibitor is broad. Thus, the inhibition activity of FXa may be a suitable measure and

a direct assay of anti-FXa activity should be developed.In addition, as shown in the XaNADU-PCI pilot trial, the measuring of whole blood INR may also provide important information regarding the effective dose (94).

5.4. Influences of direct inhibitors on platelet activation and bleeding time

The most important problem of treatment with anticoagulants is the hemorrhagic side effect. In both *in vitro* and *in vivo* experiments, most of the direct FXa inhibitors studied are considered to be a new type of antithrombotic agent with few hemorrhagic effects that do not prolong bleeding time (97, 98, 99), probably because platelet activation is not inhibited (97). Tanabe (100) evaluated the effects of DX-9065a on bleeding time and blood loss in a tail transection rat model, and on blood loss in a hydrochloride-induced gastrointestinal hemorrhage rat model. In the former, DX-9065a at concentration of 2-fold of $ID_{50}$ did not affect blood loss, whereas warfarin at a concentration of $ID_{50}$ facilitated it a level 5-fold greater than the control. In the gastrointestinal hemorrhage rat model, DX-9065a did not increase blood loss, whereas it was increased by about 2 times with warfarin. These findings suggest that the competitive and reversible inhibition of FXa by DX-9065a might result in thrombin generation sufficient to induce hemostatic plug formation, though it would be insufficient to facilitate thrombus formation (100). In a study that measured initial thrombin forming time, the time required to generate 50% thrombin activity *in vitro*, which is considered important for platelet aggregation in hemostasis, was reported to be prolonged by argatroban (1.33-fold vs.control; $p<0.002$), whereas DX-9065a and JTV-803 had no apparent influence on the initial thrombin forming time (Figure 3). Further, in the same study, platelet aggregation induced by tissue factor in defibrinated plasma was not affected by DX-9065a or JTV-803 at doses sufficient for more than 80% inhibition of thrombin generation, though it was significantly reduced by argatroban (69). These results suggest that direct inhibitors can inhibit thrombin generation significantly without affecting the activation of platelets necessary for hemostasis.

Morishima reported the evidence for the presence of a small amount of thrombin during administration of DX-9065a. Although DX-9065a inhibited dose-dependently thrombus formation in AV shunt model rats, the agent at the same concentration did not inhibit the elevation of thrombin-antithrombin complex (TAT) levels in rat plasma, whereas argatroban inhibited both thrombus formation and TAT elevation (101). DX-9065a was also reported to inhibit the formation of venous-type fibrin-rich thrombus by inactivating bound and soluble FXa without impairing platelet hemostatic function in baboons treated with an AV shunt (99). In a study with healthy volunteers, bleeding time was not prolonged when DX-9065a was administrated intravenously, even at the highest plasma concentration of 1640 ng/ml (2.87 $\mu$M). In addition, the infusion of JTV-803 at 1~10 mg/kg/hour had less of an effect on bleeding time in rats (81). In other studies, KFA-1411 did not inhibit platelet aggregation at a concentration that showed anticoagulant action, in contrast to argatroban, heparin, and HMW-heparin (dalteparin), which inhibited thrombin-induced platelet aggregation (83). In another study, bleeding times in rats and rabbits were not significantly affected at antithrombotic doses (3 mg/kg, orally, AV shunt models) of BAY59-7939 (84).

Most of the existing FXa is inhibited by FXa inhibitors, however, a small amount remains unaffected, which may bring about the small generation of thrombin that can consequently activate factor XI independently of factor XII (102, 103) and accelerate the intrinsic coagulation reaction. Further, there is a small amount of thrombin in blood that seems to be generated by factor XIa, which is activated automatically (102, 103) and/or by activated factor VII through an unknown mechanism (104). A small amount of thrombin generated by the above mechanisms seems to activate platelets, resulting in a normal bleeding time. Additionally, platelet aggregation induced by tissue factor was not affected by the presence of synthetic direct FXa inhibitors, in contrast to argatroban, which strongly inhibited aggregation (69).

## 6. CONCLUSION

The generation of thrombin is a crucial step in the process of blood coagulation, and thrombosis results from a series of proteolytic activating reactions that are initiated via intrinsic and extrinsic pathways in the blood coagulation cascade. FXa is a serine protease positioned at the convergence of those pathways and its inactivation by a specific FXa inhibitor will effectively prevent the generation of thrombin without affecting existing thrombin levels. Therefore, a sufficient level of thrombin likely remains to allow platelet activation and normal hemostasis, while preventing pathological thrombus formation.

Agents that can inhibit the activity of FXa are promising for their use in preventing thrombosis, in place of thrombin inhibitors or other anticoagulants such as warfarin. Recent therapeutic strategies for thrombosis include injectable agents such as LMW-heparin, heparinoid, and synthetic pentasaccharide, which have been reported to be specific for FXa and offer advantages over UFH. Among those, LMW heparin is distinctly superior to UFH for prophylaxis and treatment of thromboembolism, based on the results of *in vitro* studies and clinical trials. However, the most valuable and desirable antithrombotic agent is an orally active drug with broad range of effective dosages and without hemorrhagic side effects. Since orally active alternatives are better for long-term treatment of both venous and arterial thromboembolism patients, oral administration is a goal of antithrombotic drug discovery. Thus, LMW heparin and heparinoid as well as synthetic pentasaccharide are slightly inferior to synthetic selective direct FXa inhibitors such as DX-9065a. However, the efficacy of synthetic direct inhibitors for prophylaxis and treatment of thromboembolism is not clear as compared with other FXa inhibitors, because of the few clinical trials conducted thus far. On the other hand, combinations of these inhibitors and other agents such as aspirin have been reported to be effective for thromboembolism prophylaxis, though future examinations are necessary.

In conclusion, the use of an orally bioavailable FXa inhibitor alone or in combination with

antiplatelet agents is anticipated to have a significant impact on the treatment of thromboembolic disorders. Further, synthetic direct FXa inhibitors are expected to be useful for the prevention of thromboembolism in place of vitamin K antagonists.

## 7. REFERENCES


1. Davie E.W., Fujikawa K. & Kisiel W.: The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 30, 10363-10370 (1991)
2. Mann K.G., Burenas S. & Brummel K.: The dinamics of thrombin formation. *Arterioscler Throm Vasc Biol* 23, 17-25 (2003)
3. Walenga J.M., Jeske W..P., Hoppensteadt D. & Fareed J.: Factot Xa inhibitors: Today and beyond. *Curr Opin Investig Drug* 4, 272-281 (2003)
4. Samama M.M.: Synthetic direct and indirect Factor Xa inhibitors. *Thromb Res* 106: V267-V273 (2002)
5. Kaiser B.: Factor Xa – A promising target for drug development. *Cell Mol Life Sci* 59: 189-192 (2002)
6. Rydberg E.J., Westfall J.M. & Nicholas R.A.: Low-molecular-weight heparin in preventing and treating DVT. *Am Fam Physician* 59, 1607-1617 (1999)
7. Warkentin T.E., Levine M.N., Hirsh J., Horsewood P., Roberts R.S., Gent M. & Kelton J.: Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin of unfractionated heparin. *N Engl J Med* 332, 1330-1335 (1995)
8. Warkentin T.E.: Heparin-induced thrombocytopenia: a clinicopathologic syndrome. *Thromb Haemost* 82: 439-447 (1999)
9. Fragmin during instability in coronary artery disease (FRISC) study group: Low-molecular weight heparin during instability in coronary artery disease. *Lancet* 347, 561-568 (1996)
10. Klein W., Buchwald S., Hillis S., Monrad S., Sanz G., Tupie A., Van der Meer J., Olaisson E., Undeland S. & Ludwig K.: Comparison of low molecular weight heparin with UFH acutely and with placebo for 6 weeks in the management of unstable coronary artery disease: the Fragmin in Unstable Coronary Artery Disease Study (FRIC). *Circulation* 96, 61-68 (1997)
11. Cohen M., Demers C., Gurfinkel E.P., Turpie A.G.G., Fromell G.J., Goodman S., Langer A., Califf R.M., Fox K.A.A., Premmereur J. & Bigonzi F.: A comparison of low-molecular-weight heparin with unfractionated heparin for unstable coronary artery disease. *N Engl J Med* 337, 447-452 (1997)
12. Goodman S.G., Fitchett D., Armstrong P.W., Tan M. & Langer A.: Randomized evaluation of the safety and efficacy of enoxaparin versus unfractionated heparin in high-risk patients with non-ST-segment elevation acute coronary syndrome receiving the glycoprotein IIb/IIIa inhibitor eptifibatide. *Circulation* 107, 238-244 (2003)
13. THRIFT consensus group. Risk of prophylaxis for venous thromboembolism in hospital patients. *BMJ* 305, 567-574 (1992)
14. Goghaber S.Z. & Tapson V.F.: A prospective registry of 5,451 patients with ultrasound-confirmed deep vein thrombosis. *Am J Cardiol* 93, 259-262 (2004)
15. Geerts W., Cook D., Selby R. & Etchells E.: Venous thromboembolism and its prevention in critical care. *J Crit Care* 17, 95-104 (2002)
16. Turpie A.G.& Norris T.M.: Thromboprophylaxis in medical patients: the role of low-molecular-weight heparin. *Thromb Haemost* 92, 3-12 (2004)
17. Samama M.M., Cohen A.T., Darmon J-Y., Desjardins L., Eldor A., Janbon C., Leizorovicz A., Nguyen H., Olsson C-G., Turpie A.G. & Weisslinger N.: A comparison of enoxaparin with placebo for prevention of venous thromboembolism in acutely ill medical patients. *N Engl J Med* 341, 793-800 (1999)
18. Leizorovicz A., Cohen A.T., Turpie A.G.G., Olsson C-G., Vaitkus P.T. & Goldhaber S.Z.: Randomized, placebo-controlled trial of dalteparin for the prevention of venous thromboembolism in acutely ill medical patients. *Circulation* 110, 874-879 (2004)
19. Kucher N., Leizorovicz A., Vaitkus P.T., Cohen A.T., Turpie A.G.G., Olsson C-G. & Goldhaber S.Z.: Efficacy and safety of fixed low-dose dalteparin in preventing venous thromboembolism among obese or elderly hospitalized patients. *Arch Intern Med* 165, 341-345 (2005)
20. Key R., Wong K.S., Yu Y.L., Chan Y.W., Tsoi T.H., Ahuja A.T., Chan F.L., Fong K.Y., Law C.B., Wong A. & Woo J.: Low-molecular–weight heparin for the treatment of acute ischemic stroke. *N Engl J Med* 333, 1588-1593 (1995)
21. The HAEST study group: Low-molecular-weight heparin versus aspirin in patients with acute ischemic stroke and arterial fibrillation: a double-blind randomized study. *Lancet* 355, 1205-1210 (2000)
22. Diener H.C., Ringelstein E.B., von Kummer R., Langohr H.D., Bewermeyer H., Landgraf H., Hennerici M., Welzel D., Gräve M., Brom J. & Weidinger G.: Treatment of acute ischemic stroke with the low-molecular-heparin certoparin. *Stroke* 32, 22-29 (2001)
23. Danhof M., de Boer A., Magnani H. & Stiekema J.C.: Pharmacokinetic considerations on Orgaran (Org 10172) therapy. *Haemostasis* 22, 73-84 (1992)
24. Ibbotson T. & Perry C.M.: Danaparoid: A review of its use in thromboembolic and coagulation disorders. *Drug* 62, 2283-2314 (2002)
25. Mozurkewich E.L.: Preventing postsurgical deep venous thrombosis. *Female Patient Ob Gyn Ed* 22, 66-72 (1997)
26. Gent M., Hirsh J., Ginsberg J.S., Powers P.J., Levine M.N., Geerts W.H., Jay R.M., Leclerc J., Neemeh J.A. & Turpie A.G.: Low-molecular-weight heparinoid orgaran is more effective than aspirin in the prevention of venous thromboembolism after surgery for hip fracture. *Circulation* 93, 80-84 (1996)
27. Gerhart T.N., Yett H.S., Robertson L.K., Lee M.A., Smith M. & Salzman E.W.: Low-molecular-weight heparinoid compared with warfarin for prophylaxis of deep-vein thrombosis in patients who are operated on for fracture of the hip. A prospective, randomized trial. *J Bone Joint Surg Am* 73, 494-502 (1991)
28. Comp P.C., Voegeli T., McCutchen J.W., Skoutakis V.A., Trowbridge A. & Overdyke W.L.: A comparison of danaparoid and warfarin for prophylaxis against deep vein thrombosis after total hip replacement: The Danaparoid Hip Arthroplasty Investigators Group. *Orthopedics* 21, 1123-1128 (1998)
29. Bergqvist D., Kettunen K., Fredin H., Fauno P., Suomalainen O., Soimakallio S., Karjalainen P., Cederholm C., Jensen L.J., Justesen T. & Stiekema J.C.J.: Thromboprophylaxis in patients with hip fractures: a prospective, randomized, comparative study between Org 10172 and dextran 70. *Surgery* 109, 617-22 (1991)
30. Leyvraz P., Bachmann F., Bohnet J., Breyer H.G., Estoppey D., Haas S., Hochreiter J., Jakubek H., Mair J.,

Sorensen R. & Stiekema J.: Thromboembolic prophylaxis in total hip replacement: a comparison between the low molecular weight heparinoid Lomoparan and heparin-dihydroergotamine. *Br J Surg* 79, 911-914 (1992)
31. Hoek J.A., Nurmohamed M.T., Hamelynck K.J., Marti R.K., Knipscheer H.C., ten Cate H., Buller H.R., Magnani H.N. & ten Cate J.W.: Prevention of deep vein thrombosis following total hip replacement by low molecular weight heparinoid. *Thromb Haemost* 67, 28-32 (1992)
32. Cade J.F., Wood M., Magnani H.N. & Westlake G.W.: Early clinical experience of a new heparinoid, Org 10172, in prevention of deep venous thrombosis. *Thromb Res* 45, 497-503 (1987)
33. Gallus A., Cade J., Ockelford P., Hepburn S., Maas M., Magnani H., Bucknall T., Stevens J. & Porteous F.: Orgaran (Org 10172) or heparin for preventing venous thrombosis after elective surgery for malignant disease? A double-blind, randomised, multicentre comparison.ANZ-Organon Investigators' Group. *Thromb Haemost* 70, 562-567 (1993)
34. Lensing A.W.A.: Anticoagulation in acute ischemic stroke: deep vein thrombosis prevention and long-term stroke outcomes. *Blood Coagul Fibrinolysis* 10(Pt 2), 123-127 (1999)
35. The publications committee for the trial of ORG 10172 in acute stroke treatment (TOAST) investigators.Low molecular weight heparinoid, ORG 10172 (danaparoid), and outcome after acute ischemic stroke. *JAMA* 279, 1265-1272 (1998)
36. Popma J.J., Ohman E.M., Weitz J., Lincoff A.M., Harrington R.A. & Berger P.: Antiplatelet therapy in patients undergoing precutaneous coronary intervention. *Chest* 119, 321S-336S (2001)
37. Amiral J., Bridey F., Wolf M., Boyer-Neumann C., Fressinaud E., Vissac A.M., Peynaud-Debayle E., Dreyfus M. & Meyer D.: Antibodies to macromolecular platelet factor 4-heparin complexes in heparin-induced thrombocytopenia: a study of 44 cases. *Thromb Haemost* 73, 21-28 (1995)
38. Magnani H.N.: Orgaran (danaparoid sodium) use in the syndrome of heparin-induced thrombocytopenia. *Platelets* 8, 74-81 (1997)
39. Chong B.H., Gallus A.S., Cade J.F., Magnani H., Manoharan A., Oldmeadow M., Arthur C., Rickard K., Gallo J., Lloyd J., Seshadri P. & Chesterman C.N.: Prospective randomised open-label comparison of danaparoid with dextran 70 in the treatment of heparin-induced thrombocytopaenia with thrombosis: a clinical outcome study. *Thromb Haemost* 86, 1170-1175 (2001)
40. Farner B., Eichler P., Kroll H. & Greinacher A.: A comparison of danaparoid and lepirudin in heparin-induced thrombocytopenia. *Thromb Haemost* 85, 950-957 (2001)
41. Schenk J.F., Pindur G., Stephan B., Morsdorf S., Mertzlufft F., Kroll H., Wenzel E. & Seyfert U.T.: On the prophylactic and therapeutic use of danaparoid sodium (Orgaran) in patients with heparin-induced thrombocytopenia. *Clin Appl Thromb Hemast* 9, 25-32 (2003)
42. Kodityal S., Manhas A.H., Udden M. & Rice L.: Danaparoid for heparin-induced thrombocytopenia: an analysis of treatment failures. *Eur J Haematol* 71, 109-113 (2003)
43. Boneu B., Necciari J., Cariou R., Sie P., Gabaig A.M., Kieffer G., Dickinson J., Lamond G., Moelker H., Mant T. & Magnani H.: Pharmacokinetics and tolerance of the natural pentasaccharide (SR90107/Org31540) with high affinity to antithrombin III in man. *Thromb Haemost* 74, 1468-1473 (1995)
45. Leone G., Rossi E., Leone A.M. & De Stefano V.: Novel antithrombotic agents: indirect synthetic inhibitors of factor Xa and direct thrombin inhibitors. Evidences from clinical studies. *Curr Med Chem* 2, 311-326 (2004)
46. Bauersachs R.M.: Fondaparinux: an update on new study results. *Eur J Clin Invest* 35 (Supple.1), 27-32 (2005)
47. Herbert J.M., Herault J.P., Bernat A., van Amsterdam R.G.M., Lormeau J.C., Petitou M., van Boeckel C., Hoffmann P. & Meuleman D.G.: Biological and pharmacological properties of SANORG 34006, a potent and long-acting synthetic pentasaccharide. *Blood* 91, 4197-4205 (1998)
48. The persist investigators: A novel long-acting synthetic factor Xa inhibitor (SanOrg34006) to replace warfarin for secondary prevention in deep vein thrombosis. A phase II evaluation. *J Thromb Haemost* 2, 47-53 (2004)
49. Bal dit Sollier C., Kang C., Berge N., Herault J.P., Bonneau M., Herbert J.M. & Drouet L.: Activity of a synthetic hexadecasaccharide (SanOrg123781A) in a pig model of arterial thrombosis. *J Thromb Haemost* 2, 925-230 (2004)
50. Herbert J.M., Herault J.P., Bernat A., Savi P., Schaeffer P., Driguez P.A., Duchaussoy P. & Petitou M.: SR123781A, a synthetic heparin mimeric. *Thromb Haemost* 85, 852-860 (2001)
51. Turpie A.G.G., Gallus A.S. & Hoek J.A.: A synthetic pentasaccharide for the prevention of deep–vein thrombosis after total hip replacement. *N Engl J Med* 344, 619-625 (2001)
52. Turpie A.G.G., Eriksson B.I., Bauer K.A. & Lassen M.R.: New pentasaccharides for the prophylaxis of venous thromboembolism. *Chest* 124, 371S-378S (2003)
53. Cohen A.T., Davidson B.L., Gallus A.S., Lassen M.R., Tomkowski W., Turpie A.G.G., Cariou R.G., Egberts J.F.M. & Lensing A.W.A.: Fondaparinux for the prevention of venous thromboembolism in acutely ill medical patients. *Blood* 102, abstract 42 (2003)
54. Agnelli G., Bergqvist D., Cohen A., Gallus A.S. & Gent M.: A randomized doubl-blind study to compare the efficacy and safety of postoperative fondaparinux (Arixtra®) and preoperative dalteparin in the prevention of venous thromboembolism after high-risk abdominal surgery: the PEGASUS Study. *Blood* 102, abstract 40 (2003)
55. Büller H.R., Davidson B.L., Decousus H., Gallus A., Gent M., Piovella F., Prins M.H., Raskob G., Segers A.E., Cariou R., Leeuwenkamp O. & Lensing A.W.: Subcutaneous fondaparinux vs.intravenous unfractionated heparin in the initial treatment of pulmonary embolism. *Ann Intern Med* 140, 867-873 (2004)
56. The Matisse Investigators: Subcutaneous fondaparinux versus intravenous unfractinated heparin in the initial treatment of pulmonary embolism. *N Engl J Med* 349, 1695-1702 (2003)
57. Bijsterveld N.R., Moons A.H., Boekholdt S.M., van Aken B.E., Fennema H., Peters R.J.G., Meijer J.C.M., Büller H.R. & Levi M.: Ability of recombinant factor VIIa to reverse the anticoagulant effect of the pentasaccharide fondaparinux in healthy volunteers. *Circulation* 106, 2550-2554 (2002)

58. Coussement P.K., Bassand J-P., Convens C., Vrolix M., Boland J., Grollier G., Michels R., Vahanian A., Vanderheyden M., Rupprecht H.J. & van de Werf F.: A synthetic factor-Xa inhibitor (ORG31540/SR90107A) as an adjunct to fibrinolysis in acute myocardial infarction. The PENTALYSE study. *Eur Heart J* 22, 1716-1724 (2001)
59. Mehta S.R., Steg P.G., Granger C.B., Bassand J-P., Faxon D.P., Weitz J.I., Afzal R., Rush B., Peters R.J.G., Natarajan M.K., Velianou J.L., Goodhart D.M., Labinaz M., Tanguay J-F., Fox K.A.A. & Yusuf S.: Randomized, blinded trial comparing fondaparinux with unfractionated heparin in patients undergoing contemporary percutaneous coronary intervention. Arixtra study in percutaneous coronary intervention: a randomized evaluation (ASPIRE) pilot trial. *Circulation* 111, 1390-1397 (2005)
60. Vuillemenot A., Schiele F., Meneveau N., Claudel S., Donat F., Fontecave S., Cariou R., Samama M.M. & Bassand J.P.: Efficacy of a synthetic pentasaccharide, a pure factor Xa inhibitor, as an antithrombotic agent. A pilot study in the setting of coronary angioplasty. *Thromb Haemost* 81, 214-220 (1996)
61. Eriksson B.I. & Lassen M.R.: Duration of prophylaxis against venous thromboembolism with fondaparinux after hip fracture surgery: a multiceter, randomized, placebo-controlled, double-blind study. *Arch Intern Med* 163, 1337-1342 (2003)
62. Amiral J., Lormeau J.C., Marfaing-Koka A., Vissac A.M., Wolf M., Boyer-Neumann C., Tardy B., Herbert J.M. & Meyer D.: Absence of cross-reactivity of SR91107A/ORG31540 pentasaccharide with antibodies to heparin-PF4 complexes developed in heparin-induced thrombocytopenia. *Blood Coagul Fibrinolysis* 8, 114-117 (1997)
63. Amad S., Jeske W.P., Walenga J.M., Hoppensteadt D.A., Wood J.J., Herbert J.M., Messmore H.L. & Fareed J.: Synthetic pentasaccharides do not cause platelet activation by antiheparin-platelet factor 4 antibodies. *Cli Appl Thromb Hemost* 5, 259-266 (1999)
64. Savi P., Chong B.H., Greinacher A., Gruel Y., Kelton J.G., Warkentin T.E., Eichler P., Meuleman D., Petitou M., Herault J-P., Cariou R. & Herbert J-M.: Effect of fondaparinux on platelet activation in the presence of heparin-dependent antibodies: a blinded comparative multicenter study with unfractionated heparin. *Blood* 105, 139-144 (2005)
65. Hara T., Yokoyama A., Ishihara H., Yokoyama Y., Nagahara T. & Iwamoto M.: DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. *Thromb Haemost* 71, 314-319 (1994)
66. Rezaie A.R.: DX-9065a inhibition of factor Xa and the prothrombinase complex: mechanism of inhibition and comparison with therapeutic heparins. *Thromb Haemost* 89, 112-121 (2003)
67. Dyke C.K., Becker R.C., Kleiman N.S., Hochman J.S., Bovill E.G., Lincoff A.M., Gerstenblith G., Dzavik V., Gardner L.H., Hasselblad V., Zillman L.A., Shimoto Y., Robertson T.L., Kunitada S., Armstrong P.W. & Harrington R.A.: First experience with direct factor Xa inhibitor in patients with stable coronary disease. *Circulation* 105, 2385-2391 (2002)
68. Murayama N., Tanaka S., Kikuchi T., Nakaoka M. & Sudo K-I.: Radioimmunoassay method for DX-9065a, an anticoagulant agent. Development, evaluation and appication to human plasma. *J Pharm Biomed Anal* 14, 1435-1445 (1996)
69. Ieko M., Tarumi T., Takeda M., Naito S., Nakabayashi T. & Koike T.: Synthetic selective inhibitors of coagulation factor Xa strongly inhibit thrombin generation without affecting initial thrombin forming time necessary for platelet activation in hemostasis. *J Thromb Haemost* 2, 612-618 (2004)
70. Becker R.C., Alexander J., Li Y.F., Bovill E., Spencer F.A., Robertson T.L., Kunitada S., Dyke C.K. & Harrington R.A.: Combined coagulation phase-directed factor Xa inhibition with heparin compounds and DX-9065a - a direct and selective antagonist. *Thromb Haemost* 92, 1229-1231 (2004)
71. Brandstetter H., Kühne A., Bode W., Huber R., von der Saal W., Wirthensohn K. & Engh R.A.: X-ray structure of active site-inhibited clotting factor Xa. Implications for drug design and substrate recognition. *J Biol Chem* 271, 29988-29992 (1996)
72. Shimbo D., Osende J., Chen J., Robbins J., Shimoto Y., Kunitada S., Fuster V. & Badimon J.J.: Antithrombotic effects of DX-9065a, a direct factor Xa inhibitor: a comparative study in humans versus low molecular weight heparin. *Thromb Haemost* 88, 733-738 (2002)
73. Herault J.P., Perrin B., Jongbloet C., Pflieger A.M., Bernat A. & Herbert J.M.: Effect of factor Xa inhibitors on the platelet-derived microparticles procoagulant activity *in vitro* and *in vivo* in rats. *Thromb Haemost* 84, 668-674 (2000)
74. Shu E., Matsuno H., Ishisaki A., Kitajima Y. & Kozawa O.: Lack of plasminogen activator inhibitor-1 enhances the preventive effect of DX-9065a, a selective factor Xa inhibitor, on venous thrombus and acute pulmonary embolism in mice. *Pathophysiol Haemaost Thromb* 33, 206-213 (2003)
75. Tanabe K., Terada Y., Shibutani T., Kunitada S. & Kondo T.: A specific inhibitor for factor Xa, DX-9065a, exerts effective protection against experimental tumor-induced disseminated intravascular coagulation in rats. *Thromb Res* 96, 135-143 (1999)
76. Taniuchi Y., Sakai Y., Hisamichi N., Kayama M., Mano Y., Sato K., Hirayama F., Koshio H., Matsumoto Y. & Kawasaki T.: Biochemical and pharmacological characterization of YM-60828, a newly synthesized and orally active inhibitor of human factor Xa. *Thromb Haemost* 79, 543-548 (1998)
77. Kawasaki T., Sato K., Sakai Y., Hirayama F., Koshio H., Taniuchi Y. & Matsumoto Y.: Comparative studies of an orally-active factor Xa inhibitor, YM-60828, with other antithrombotic agents in a rat model of arterial thrombosis. *Thromb Haemost* 79, 410-416 (1998)
78. Mano Y., Sonoda T., Nakamura E., Usui T. & Kamimura H.: Absorption, Distribution, Metabolim and excretion of YM466, a novel factor Xa inhibitor, in rats. *Biopharm Drug Dispos* 25, 253-260 (2004)
79. Iwatsuki Y., Kawasaki T., Hayashi K., Moritani Y., Nii T. & Miyata K.: Combined effects of a factor Xa inhibitor YM466 and a GPIIb/IIIa antagonist YM128 on thrombosis and neointima formation in mice. *Thromb Haemost* 92, 1221-1228 (2004)
80. Hayashi M., Hamada A., Okaya Y., Wakitani K. & Aisaka K.: Inhibition effect of JTV-803, a new cyclic guanidine derivatives, on factor Xa *in vitro* and *in vivo*. *Eur J Pharmacol* 428, 163-168 (2001)
81. Hayashi M., Mastuo A., Nakamoto H. & Aisaka K.: Antithrombotic effects of a synthetic inhibitor of activated factor X, JTV-803, in animals. *Eur J Pharmacol* 412, 61-66 (2001)

82. Asakura H., Ichino T., Yoshida T., Suga Y., Ontachi Y., Mizutani T., Kato M., Ito T., Yamazaki M., Aoshima K., Morishita E., Saito M., Miyamoto K.I. & Nakao S.: Beneficial effect of JTV-803, a new synthetic inhibitor of activated factor X, against both lipopolysaccharide-induced and tissue factor-induced disseminated intravascular coagulation in rat models. *Blood Coagul Fibrinolysis* 13, 233-239 (2002)
83. Hara K., Honma T., Matsuzawa A., Matsuzawa A., Uchida M., Koizumi T., Akahane S. & Kojima M.: Characteristics of the hemostatic action of KFA-1411, an inhibitor of coagulation factor Xa (Xa), in humans and various animals. *J Toxicol Sci* 28, 25-34 (2003)
84. Perzborn E., Strassburger J., Wilmen A., Pohlmann J., Roehrig S., Schlemmer K.H. & Straub A.: *In vitro* and *in vivo* studies of the novel antithrombotic agent BAY 59-7939--an oral, direct Factor Xa inhibitor. *J Thromb Haemost* 3, 514-521 (2005)
85. Kubitza D., Becka M., Wensig G., Voith B. & Zuehlsdorf M.: Multiple dose escalation study investigating the pharmacodynamics, safety, and pharmacokinetics of BAY 59-7939 an oral, direct factor Xa inhibitor in healthy male subjects. *Blood* 102, abstract 3004 (2003)
86. Pezborn E.P., Strassburger J., Wilmen A., Lampe T., Pernerstorfer P., Pohlmann J., Roehrig S., Schlemmer K-H. & Straub A.: Biochemical and pharmacologic properties of BAY 59-7939, an oral, direct factor Xa inhibitor. *Pathophysiol Haemost Thromb* 33 (Suppl.2), abstract PO079 (2004)
87. Kubitza D., Becka M., Wensig G., Voith B. & Zuehlsdorf M.: Single dose escalation study investigating the pharmacodynamics, safety, and pharmacokinetics of BAY 59-7939 an oral, direct factor Xa inhibitor in healthy male subjects. *Blood* 102, abstract 3010 (2003)
88. Wong P.C., Crain E.J., Watson C.A., Zaspel A.M., Wright M.R., Lam P.Y., Pinto D.J., Wexler R.R. & Knabb R.M.: Nonpeptide factor Xa inhibitors III: effects of DPC423, an orally-active pyrazole antithrombotic agent, on arterial thrombosis in rabbits. *J Pharmacol Exp Ther* 303, 993-1000 (2002)
89. Quan M.L., Lam P.Y.S., Han Q., Pinto D.J.P., He M.Y., Li R., Ellis C.D., Clark C.G., Teleha C.A., Sun J.H., Alexander R.S., Bai S., Luettgen JM., Knabb R.M., Wong P.C. & Wexler R.R.: Discovery of 1-(3'-aminobenzisoxazol-5'-yl)-3-trifluoromethyl-N-[2-fluoro-4-[(2'-dimethylaminomethyl)imidazol-1-yl]phenyl]-1H-pyrazole-5-carboxyamide hydrochloride (razaxaban), a highly potent, selective, and orally bioavailable factor Xa inhibitor. *J Med Chem* 48, 1729-1744 (2005)
90. Wong P.C., Watson C.A., Crain E.J., Zaspel A.M., Luettgen J.M., Bai S.T., Lam P.Y., Quan M.L., Wexler R.R. & Knabb R.: Antithrombotic effects of razaxaban, an orally-active factor Xa inhibitor, in rabbiot models of thrombosis. *Blood* 102, abstract 3011 (2003)
91. Pinto D.J., Orwat M.J., Wang S., Fevig J.M., Quan M.L., Amparo E., Cacciola J., Rossi K.A., Alexander R.S., Smallwood A.M., Luettgen J.M., Liang L., Aungst B.J., Wright M.R., Knabb R.M., Wong P.C., Wexler R.R. & Lam P.Y.: Discovery of 1-[3-(aminomethyl)phenyl]-N-3-fluoro-2'-(methylsulfonyl)-[1,1'-biphenyl]-4-yl]-3-(trifluoromethyl)-1H-pyrazole-5-carboxamide (DPC423), a highly potent, selective, and orally bioavailable inhibitor of blood coagulation factor Xa. *J Med Chem* 44, 566-578 (2001)
92. Wong G.C., Giugliano R.P. & Antman E.M.: Use of low-molecular-weight heparins in the management of acute coronary artery syndromes and percutaneous coronary intervention. *JAMA* 289, 331-342 (2003)
93. Simoons M.L., Bobbink I.W., Boland J., Gardien M., Klootwijk P., Lensing A.W., Ruzyllo W., Umans V.A., von De Werf F. & Zeymer U.: A dose-finding study of fondaparinux in patients with non-ST-segment elevation acute coronary syndrome: the Pentasaccharide in Unstable Angina (PENTUA) Study. *J Am Coll Cardiol* 43, 2183-2190 (2004)
94. Alexander J.H., Dyke C.K., Yang H., Becker R.C., Hasselblad V., Zillman L.A., Kleiman N.S., Hochman J.S., Berger P.B., Cohen E.A., Lincoff A.M., Saint-Jacques H., Chetcuti S., Burton J.R., Buergler J.M., Spence F.P., Shimoto Y., Robertson T.L., Kunitada S., Bovill E.G., Armstrong P.W. & Harrington R.A.: Initial experience with factor-Xa inhibition in percutaneous coronary intervention: the XaNADU-PCI Pilot. *J Thromb Haemost* 2, 234-241 (2004)
95. Alexander J.H., Yang H., Becker R.C., Kodama K., Goodman S., Dyke C.K., Kleiman N.S., Hochman J.S., Berger P.B., Cohen E.A., Lincoff A.M., Burton J.R., Bovill E.G., Kawai C., Armstrong P.W. & Harrington R.A.: First experience with direct, selective factor Xa inhibition in patients with non-ST-elevation acute coronary syndromes: results of the XaNADU-ACS Trial. *J Thromb Haemost* 3, 439-447 (2005)
96. Lassen M.R., Davidson B.L., Gallus A., Pineo G., Ansell J. & Deitchman D.: A phase II randomized, double-blind, five-arm, parallel-group, dose-response study of a new oral directly-acting factor Xa inhibitor, Razaxaban, for the prevention of deep vein thrombosis in knee replacement surgery. *Blood* 102, abstract 41 (2003)
97. Yamazaki M., Asakura H., Aoshima K., Saito M., Jokaji H., Uotani C., Kumabashiri I., Morishita E., Ikeda T. & Matsuda T.: Effects of DX-9065a, an orally active, newly synthesized and specific inhibitor of factor Xa, against experimental disseminated intravascular coagulation in rats. *Thromb Haemost* 72, 392-396 (1994)
98. Hara T., Yokoyama A., Tanabe K., Ishihara H. & Iwamoto M.: DX-9065a, an orally active, specific inhibitor of factor Xa, inhibits thrombosis without affecting bleeding time in rats. *Thromb Haemost* 74, 635-639 (1995)
99. Yokayama T., Kelly A.B., Marzec U.M., Hanson S.R., Kunitada S. & Harker L.A.: Antithrombotic effects of orally active synthetic antagonist of activated factor X in nonhuman primates. *Circulation* 92, 485-491 (1995)
100. Tanabe K., Morishima Y., Shibutani T., Terada Y., Hara T., Shinohara Y., Aoyagi K., Kunitada S. & Kondo T.: DX-9065a, an orally active factor Xa inhibitor, does not facilitate haemorrhage induced by tail transection or gastric ulcer at the effective doses in rat thrombosis model. *Thromb Haemost* 81, 828-834 (1999)
101. Morishima Y., Tanabe K., Terada Y., Hara T. & Kunitada S.: Antithrombotic and hemorrhagic effects of DX-9065a, a direct and selective factor Xa inhibitor: comparison with a direct thrombin inhibitor and antithrombin III-dependent anticoagulants. *Thromb Haemost* 78, 1366-1371 (1997)
102. Naito K. & Fujikawa K.: Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. *J Biol Chem* 266, 7353-7358 (1991)
103. Gailani D. & Broze G.J.Jr.: Factor XI activation in a revised model of blood coagulation. *Science* 253, 909-912 (1991)

104. Hemker H.C.: Thrombin generation, an essential step in haemostasis and thrombosis. In: Haemostasis and Thrombosis Vol.1, 3rd edn.Eds: Bloom A.L., Forbes C.D.Thomas D.P. & Tuddenham E.G.D. *Churchill Livingstone*, Edinburgh 477-490 (1994)
105. Eriksson B.I., Bauer K.A., Lassen M.R. & Turpie A.G.G.: Fondaparinux compared with enoxaparin for the prevention of venous thromboembolism after hip fracture surgery. *N Engl J Med* 345, 1298-1304 (2001)
106. Bauer K.A., Eriksson B.I., Lassen M.R. & Turpie A.G.G.: Fondaparinux compared with enoxaparin for the prevention of venous thromboembolism after elective major knee surgery. *N Engl J Med* 345, 1305-1310 (2001)
107. Lassen M.R., Bauer K.A., Eriksson B.I. & Turpie A.G.G.: Postoperative fondaparinux versus preoperative enoxaparin for prevention of venous thromboembolism in elective hip replacement surgery: a randomized double-blind trial. *Lancet* 356, 1715-1720 (2002)
108. Turpie A.G.G., Bauer K.A., Eriksson B.I. & Lassen M.R.: Postoperative fondaparinux versus postoperative enoxaparin for prevention of venous thromboembolism after elective hip-replacement surgery: a randomized double-blind trial. *Lancet* 359, 1721-1726 (2002)

Abbreviations: ACS: acute coronary syndrome, aPTT: activated partial thromboplastin time, AT: antithrombin, AV shunt: arterio-venous shunt, DIC: disseminated intravascular coagulation, DVT: deep-vein thrombosis, HIT: heparin-induced thrombocytopenia, INR: international normalized ratio, LMW heparin: low molecular weight heparin, PCI: percutaneous coronary intervention, PE: pulmonary embolism, PT: prothrombin time, rt-PA: recombinant tissue-plasminogen activator, TAT: thrombin-antithrombin complex, UFH: unfractionated heparin, VTE: venous thromboembolism.

Key Words: Factor Xa inhibitor, Heparin, Heparinoid, Pentasaccharide, Thromboembolism, Review

Send correspondence to: Masahiro Ieko, MD, PhD, Department of Internal Medicine, School of Dentistry, Health Sciences University of Hokkaido, 1757-Kanazawa, Ishikari-Tobetsu, Hokkaido, 061-0293, Japan, Tel: 81-133-23-3070, Fax: 81-133-23-1534, E-mail: iekom@hoku-iryo-u.ac.jp

http://www.bioscience.org/current/vol11.htm

Journal of Biological Sciences 6 (1): 150-159, 2006
ISSN 1727-3048


# TRAIL-mediated Cytotoxicity: Impacts of sTRAIL and vTRAIL Microvesicles

[1]Akira Furusaki, [1,2]Satoshi Jodo, [1]Yumi Yamashita, [1]Yoshiharu Amasaki, [1]Tatsuya Atsumi and [1]Takao Koike
[1]Department of Medicine II, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan
[2]Department of Medicine, Kitami Red Cross Hospital, Kita 6, Higashi 2-1, Kitami, Hokkaido 090-8666, Japan

**Abstract:** Previous studies have shown that FasL-expressing cells produced nearly equal amount of soluble FasL (sFasL) and microvesicle-associated FasL (vFasL) under regular tissue culture condition. Here, we studied the ability of TRAIL-expressing cells to produce sTRAIL and vTRAIL and compared their impact on TRAIL-mediated cytotoxicity. We found that TRAIL-expressing cells produced extremely low level of vTRAIL. It indicates that the ability of TRAIL-expressing cells to produce vTRAIL but not sTRAIL is significantly different from FasL-expressing cells and that the ability to produce vTRAIL and vFasL is a property intrinsic to the protein itself. Our study also shows that the microvesicles, containing full-length TRAIL, express strong cytotoxicity against a commonly used Jurkat target cells whereas the cytotoxicity of sTRAIL was nearly undetectable. We concluded that sTRAIL is efficiently produced so much so that it can be inhibitory for the cytotoxicity expressed by the TRAIL-expressing cells. The significance of the findings is discussed.

**Key words:** TRAIL, soluble TRAIL, TRAIL microvesicles, cytotoxicity, apoptosis

## INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II membrane protein that belongs to the TNF family[1,2]. Among members of this family, the extracellular portion of TRAIL is highly homologous to that of the CD95 ligand (FasL)[3]. Like FasL, TRAIL also induces apoptosis of target cells. In humans, TRAIL can bind to two death-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5)[4-7], leading to receptor-clustering followed by activation of the caspase-cascade and induction of apoptosis in TRAIL-sensitive cells[8-11].

TRAIL is expressed in activated human T cells[12-14], monocytes/macrophages[15], natural killer cells[16-20] and dendritic cells[21-23]. TRAIL-mediated apoptosis was reported to contribute to the activation-induced death of human peripheral blood T cells. Like the Fas/FasL system, TRAIL-based immune regulation may play a role in autoimmune diseases and the maintenance of immune privilege sites[24-28]. In addition, TRAIL is reported to preferentially kill virus-infected[25] and malignant tumor cells[1,2,6,30,31], but not normal cells[32]. Therefore, TRAIL has been proposed to be a potentially useful therapeutics. Many investigators have attempted to make agonistic recombinant soluble TRAIL as well as agonistic anti-TRAIL receptor antibodies. These reagents can be used to kill cancer cells either by themselves or in combination with anti-tumor drugs[14,33-39].

Cells that express TRAIL express killing activity against TRAIL-sensitive target cells. Like FasL-expressing cells, TRAIL-expressing cells also release a soluble form of TRAIL (sTRAIL) thought to be cleaved by protease(s)[26] that is yet to be identified. In addition to sTRAIL, human TRAIL-expressing cells also secrete microvesicles that contain TRAIL (vTRAIL) and vTRAIL are cytotoxic against TRAIL-sensitive target cells[40-42]. While production of sTRAIL and sFasL is dependent on the presence of sensitive sites on the extracellular domains of TRAIL/FasL, the requirement for the production of vTRAIL and vFasL is not well understood. In the present study, we quantitatively investigated the amount of sTRAIL and vTRAIL produced by TRAIL-expressing cells. Present study suggests that TRAIL-expressing cells preferentially secrete sTRAIL but not vTRAIL. The ability to produce vTRAIL appears to be an intrinsic property of the TRAIL itself. Moreover, the high level of sTRAIL in the culture supernatant could act as feedback inhibitor for cell-mediated, TRAIL-dependent cytotoxicity. This study characterized TRAIL turnover system and defined the functional significance of sTRAIL and vTRAIL.

## MATERIALS AND METHODS

**TRAIL-expressing cell lines:** Human TRAIL cDNA, provided by Dr. J. Tschopp, was cloned into Moloney

**Corresponding Author:** Dr. Satoshi Jodo M.D., Department of Medicine, Kitami Red Cross Hospital, Kita 6, Higashi 2-1, Kitami, Hokkaido, 090-8666, Japan Tel: +81-157-24-3115 Fax: +81-157-22-3339

leukemia virus-derived pLXSN (GenBnk accession No. M28248)[42]provided by Dr. A.D. Miller. The TRAIL construct, pMLV-hTRAIL, was used to transfect the packaging cell line PE501 (a gift from Dr. A.D. Miller) using lipofectamine (GIBCO). Viral-laden supernatants from transfected cell line were recovered at 48 h after transfection and were used to infect the PA317 packaging cell line that was provided by Dr. S.T. Ju. Clones were selected with 0.75 mg $mL^{-1}$ of G418 (GIBCO) and the cell line TRAIL-PA317 that expressed the strongest cytotoxicity was used in the present study. A similarly prepared packaging cell line Krox-PA317 carrying the human *ckrox* gene[44] was used for the preparation of various controls throughout the study. The generation of both TRAIL-PA317 and Krox-PA317 cells has been described previously[42]. We also established TRAIL expressing NIH-3T3 cells by transferring the human *trail* gene to NIH-3T3 cells. Various amounts of vector prepared from packaging cells were cultured with NIH-3T3 cells ($2 \times 10^5$ cells $well^{-1}$ in 24-well plates) in the presence of 6 μg $mL^{-1}$ polybrene (Sigma). Medium was replaced 24 h later with fresh medium containing 0.75 mg $mL^{-1}$ G418. Cell populations that survived the G418 selection were examined for TRAIL-mediated cytotoxicity. One cell line (TRAIL-3T3) that expresses strongest cytotoxicity was used in this study.

**Target cells:** A20, CEM, Jurkat, HCT-15 and Hep G2 cells were purchased from ATCC. A-172, MOLT-4 and U-937 cells were provided by Dr. K. Nishimura (Division of Immunoregulation, Institute for Genetic Medicine, Hokkaido University).

**Preparation of sTRAIL and vTRAIL:** TRAIL-expressing cells (80% confluence) were maintained in 150×25 mm Petri dishes (FALCON) in 30 mL of culture media. Culture supernatants and cells were harvested 24 h later. The cell number harvested was ~ $30 \times 10^6$/dish. Supernatants were centrifuged at 5°C for 30 min at 15,000 rpm in a Beckman centrifuge (Avanti™ 30) using an F0650 rotor to remove cell debris. To prepare sTRAIL, the Cell-free Supernatants (CFS) were centrifuged at 5°C for 16 h at 25,000 rpm in a Beckman ultracentrifuge (L8-M) using a SW28 rotor. The top 10% volume was collected in order to avoid potential contamination of vesicles. This Vesicle-free Supernatant (VFS) was passed through a 0.45 μm sterile filter and stored at 4°C. The Vesicle-containing Pellet (VP) was suspended with culture medium to 3% of the original volume. The suspension was passed through a 0.45 μm sterile filter and stored at 4°C.

Filtration based on molecular size was used to characterize various TRAIL-containing fractions. The filtration/concentration apparatus used were MACROSEP 300K OMEGA (Pall Life Sciences) which retain components larger than 300 kDa and Centricon Plus-20 PL-30 (Millipore) which retains components larger than 30 kDa. The former was used for concentrating vTRAIL (present in microvesicles) and the latter was used to concentrate sTRAIL (~60 kDa as a trimer).

**Flow cytometric analysis:** TRAIL-PA317, TRAIL-3T3, Krox-PA317 and NIH-3T3 cells were incubated with 0.1 μg of Phycoerythrin (PE)-conjugated mouse anti-human TRAIL mAb (RIK-2, mouse IgG1/κ) and PE-conjugated control mouse IgG1 (DAKO) for 30 min on ice. After washing with PBS containing 0.2% BSA, the cells were analyzed on a FACS Calibur™ (Becton Dickinson) and data were processed using Cell Quest™ software (Becton Dickinson).

**Quantification of TRAIL:** Protein concentrations of TRAIL were determined using a capture ELISA (OptEIA™ Human TRAIL set, Pharmingen). This assay measures both soluble TRAIL and transmembranous TRAIL, because the mAbs used recognize epitopes present on soluble TRAIL and full-length TRAIL. To measure cellular TRAIL, cells ($4 \times 10^6$) were washed with PBS, then treated with 1 mL of Cell Lysis Buffer (Pharmingen) with Protease inhibitor Cocktail (Pharmingen), according to the instruction in the kit. VP tested in this study obtained from different cells were re-suspended and filled with PBS, then pelleted by ultracentrifugation once more, re-suspended again with PBS, then treated with Cell Lysis Buffer and Protease inhibitor Cocktail at the recommended ratio. VFS obtained from different cells were analyzed without using lysis buffer. All samples were diluted with Assay Dilute (Pharmingen) and immediately assayed. Standard curves were generated with various concentrations of recombinant TRAIL provided with the kit.

**Cytotoxicity assays:** Target cells were labeled with $Na_2{}^{51}CrO_4$ as described[45]. The effector samples included TRAIL-expressing cells, VP and VFS. Various amounts of samples were cultured with $2 \times 10^4$ target cells in a total of 0.2 mL in individual wells of a 96-well plate. In some experiments, inhibitor was added to the mixtures at the beginning of culture to determine their effect on cytotoxicity. The inhibitor used was DR5-Fc (Alexis) that consists of the extracellular domain of the human TRAIL receptor DR5 fused with the Fc portion of human IgG and it was seen to specifically block TRAIL-induced apoptosis. Supernatants of cytotoxicity assays were removed at an appropriate period after culture and