Nonpeptide Factor Xa Inhibitors II. Antithrombotic Evaluation in 
a Rabbit Model of Electrically Induced Carotid Artery 
Thrombosis

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ABSTRACT

SK549 (mol. wt. 546 Da) is a synthetic, selective inhibitor of human coagulation factor Xa (fXa) ($K_i = 0.52 \text{nM}$). This study compared the antithrombotic effects of SK549 and a series of benzamidine isoxazoline fXa inhibitors with aspirin, DuP 714 (a direct thrombin inhibitor), recombinant tick anticoagulant peptide, or heparin in a rabbit model of electrically induced carotid arterial thrombosis. Compounds were infused i.v. continuously from 60 min before electrical stimulation to the end of the experiment. Values of ED$_{50}$ (dose that increases the carotid blood flow to 50% of the control) were 0.12 $\mu$mol/kg/h for SK549, 0.56 $\mu$mol/kg/h for aspirin, 0.14 $\mu$mol/kg/h for DuP 714, 0.06 $\mu$mol/kg/h for recombinant tick anticoagulant peptide, and $>100$ U/kg/h for heparin. The EC$_{50}$ (plasma concentration that increased blood flow to 50% of the control) for SK549 was 97 nM. Unlike aspirin and heparin, SK549 was efficacious and, at 1.5 $\mu$mol/kg/h i.v. ($n = 9$), maintained carotid blood flow at 67 ± 6% of control level for greater than 90 min. Unlike heparin, SK549 inhibited ex vivo fXa activity but not ex vivo thrombin activity. There was a highly significant correlation between $K_i$ (fXa) and ED$_{50}$ of a series of fXa inhibitors ($r = 0.85, P < .001$). Therefore, these results suggest that SK549 is a novel, potent, and effective antithrombotic agent in a rabbit model of arterial thrombosis. It is likely that SK549 exerts its antithrombotic effect through selective inhibition of fXa. Furthermore, SK549 may be clinically useful for the prevention of arterial thrombosis.

The clinical usefulness of anticoagulants such as warfarin (Coumadin) and the successful development of low-molecular-weight heparin for the treatment and prevention of thromboembolic diseases have generated great interest in designing new inhibitors of blood coagulation (Turpie, 1998). The most promising new inhibitors of blood coagulation are inhibitors of thrombin or factor Xa (fXa) (Fevig and Wexler, 1999; Hauptmann and Stürzebecher, 1999). Both naturally occurring and synthetic thrombin inhibitors have been well studied for the past two decades (Hauptmann and Stürzebecher, 1999). However, it is still not clear whether the desired antithrombotic effects of these inhibitors can be achieved without undesirable bleeding complications (Turpie, 1998). Thus, there is an increasing interest in developing synthetic and selective fXa inhibitors (Fevig and Wexler, 1999; Sinha, 1999).

Similar to thrombin inhibitors, both naturally occurring and synthetic fXa inhibitors are available and have been shown to be potent antithrombotic agents in animal models of thrombosis (Wong et al., 1996; Kaiser, 1998; Hauptmann and Stürzebecher, 1999). However, in contrast to thrombin inhibitors, it is believed that inhibition of fXa may reduce the production of thrombin by either the extrinsic or intrinsic pathways without interfering with a basal level of thrombin activity necessary for normal hemostasis (Harker et al., 1997).

Recently, DuPont Pharmaceuticals has discovered an interesting novel series of potent and selective nonpeptide fXa inhibitors, exemplified by (+)-(5)-isoxazolcarboxamide, 3-[3-(aminomethyl)phenyl]-N-5-[1,1’-biphenyl]-4-yl]-4,5-dihydro-5-(1H-tetrazol-1-ylmethyl)-trifluoroacetic acid salt (SK549) (Fig. 1) (Quan et al., 1999a,b; Wong et al., 2000). SK549 is a potent and selective fXa inhibitor ($K_i$: fXa, 0.52 nM; thrombin, 400 nM; trypsin, 45 nM; tissue plasminogen activator, >33,000 nM; plasmin, 890 nM) (Wong et al., 2000). It has a low plasma clearance of 0.3
sk549 for the prevention of arterial thrombosis has not been studied. Therefore, we evaluated in this study the antithrombotic efficacy of fXa inhibitors, it may not be a very physiologically relevant model. Furthermore, the effectiveness of SK549 for the prevention of arterial thrombosis has not been studied. Therefore, we evaluated in this study the antithrombotic effect of SK549 and its chemical analogs in a rabbit model of arterial thrombosis. Thrombosis in this model was produced by electrically induced injury of the carotid artery. Because arterial thrombosis in humans usually occurs in areas of medium-to-high blood flow and shear stress with a triggering factor of vascular injury (Badimon, 1997), the model of electrical current-induced arterial thrombosis (ECAT) in rabbits may have some pathophysiological relevance to the human disease. In this study, we also included heparin, aspirin, DuP 714 (a direct thrombin inhibitor) (Kettner et al., 1990; Knabb et al., 1992), and recombinant human α-thrombin and fXa (Enzyme Research Laboratories, Inc., South Bend, IN); protease inhibitor cocktail (Complete; Boehringer Mannheim GmbH, Indianapolis, IN); human γ-thrombin (ICN Biomedicals, Inc., Costa Mesa, CA); activated partial thromboplastin time (APTT) reagent, ADP, aspirin, and thromboplastin with calcium (Sigma Chemical Co., St. Louis, MO); and heparin (Upjohn, Kalamazoo, MI). Nonpeptide fXa inhibitors were synthesized at DuPont Pharmaceuticals Company. Purified rTAP was prepared from culture medium of Saccharomyces cerevisiae as described by Neeper et al. (1990) with modifications.

Fig. 1. Structural formulae and biological activities of a series of nonpeptide fXa inhibitors where R, X, and Y are functional groups of the molecules. fXa Ki is the inhibitory constant for human fXa. ID50 is the dose that produces 50% inhibition of thrombus formation in the arteriovenous shunt thrombosis rabbits. Ki and ID50 data for the compounds listed in the table were reported in Wong et al. (2000); ED50 is the in vivo antithrombotic potency obtained in ECAT rabbits.

<table>
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<tr>
<th>Compound</th>
<th>R</th>
<th>X</th>
<th>Y</th>
<th>fXa Ki (nM)</th>
<th>ID50 (pmol/kg/h)</th>
<th>ED50 (μmol/kg/h)</th>
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<td>CH</td>
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<td>0.52</td>
<td>0.035</td>
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Materials and Methods

All experiments were conducted in accordance with the regulations of the Animal Care and Use Committee of the DuPont Pharmaceuticals Company.

Reagents. The following drugs and chemicals were used in this study: chromogenic substrates S-2222 and S-2238 (Chromogenix AB products distributed by DiaPharma Group, Inc., West Chester, OH); human α-thrombin and fXa (Enzyme Research Laboratories, Inc., South Bend, IN); protease inhibitor cocktail (Complete; Boehringer Mannheim GmbH, Indianapolis, IN); human γ-thrombin (ICN Biomedicals, Inc., Costa Mesa, CA); activated partial thromboplastin time (APTT) reagent, ADP, aspirin, and thromboplastin with calcium (Sigma Chemical Co., St. Louis, MO); and heparin (Upjohn, Kalamazoo, MI). Nonpeptide fXa inhibitors were synthesized at DuPont Pharmaceuticals Company. Purified rTAP was prepared from culture medium of Saccharomyces cerevisiae as described by Neeper et al. (1990) with modifications.

Electrically Induced Arterial Thrombosis Model in Rabbits. Experiments, using a modification of the methods of Hladovec (1971) and Guarini (1996), were conducted on male New Zealand White rabbits (2.7–3.1 kg). The rabbits were anesthetized with ketamine (50 mg/kg + 50 mg/kg i.m.) and xylazine (10 mg/kg + 10 mg/kg i.m.). The left femoral vein and artery were isolated and catheterized. Both common carotid arteries were carefully isolated. Carotid blood flow was measured with a calibrated flow probe (3.5-mm circumference) that was linked to an electromagnetic flowmeter (FM501D; Carolina Medical Electronics, Inc., King, NC). A stainless steel bipolar hook electrode was placed on the carotid artery and positioned caudally in relationship to the flow probe. A piece of Parafilm (7 × 30 mm) was placed under the electrode to protect the surrounding tissue. Thrombosis was induced by applying a direct electrical current of 4 mA for 3 min to the external arterial surface, using a constant current unit and a d.c. stimulator (SS8D; Grass Instruments Co., Quincy, MA). We chose to stimulate the carotid artery at 4 mA because we found that in a preliminary study, electrical stimulation at 1 mA for 3 min did not produce occlusive thrombus within 40 min. However, increasing the current to 4 mA produced a more reproducible occlusion of the injured carotid artery within 40 min. In this study, carotid blood flow was monitored continuously before and after electrical stimulation. The left carotid artery served as a control artery. If the left carotid artery did not occlude within 5 to 40 min after electrical stimulation, these rabbits were not included in the study. Rabbits that were excluded from the study represented about 5% of rabbits used.

After the determination of the control time to occlusion, the compound or saline was given as continuous i.v. infusion via the femoral vein, starting 1 h before the electrical stimulation and continuing to the end of the test. Thrombosis was electrically induced in the right common carotid artery, using the same method mentioned above.

When carotid blood flow was decreased to zero, the time to occlusion in minutes was noted. If the arteries were still patent at 90 min after electrical stimulation at 1 mA for 3 min, the arteries were considered patent. If the arteries occluded within 5 to 40 min after electrical stimulation, these rabbits were included in the study. Rabbits that were excluded from the study represented about 5% of rabbits used.

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Antithrombotic Studies in Arterial Thrombosis. Rabbits were dosed i.v. with saline vehicle (6 ml/kg/h), heparin (64 and 100
addition of 50 m trometry method (Wong et al., 2000). The EC50 (plasma concentra-

strates S-2222 and S-2238, respectively. Assays were performed in a
spectrophotometer (Molecular Devices, Sunnyvale, CA). All assays were performed in 96-well plates (no. 3600; Corning Inc., Corning, NY). Thirty microliters of S-2222 (500 µM) or S-2238 (500 µM) was added to a mixture of 30 µl of buffer (0.1 M Tris, pH 7.5; 0.2 M NaCl), 30 µl of platelet-poor plasma, and 30 µl of human

In some rabbits treated with SK549, concentrations of SK549 in

(mean). Scale bar, 10 µm.

Fig. 2. Scanning electron micrographs of internal side of a normal carotid artery (A) and an injured carotid artery (B) after 2-min electrical stimulation. Scale bar, 10 µm.
average carotid blood flow of 26 ± 14% of the control level (Fig. 3).

**Antithrombotic Effect of Aspirin, DuP 714, and rTAP.** Figure 4 shows effects of aspirin (2 to 56 μmol/kg/h i.v.), DuP 714 (0.06 to 0.6 μmol/kg/h i.v.), and rTAP (0.03 and 0.05 μmol/kg/h i.v.) on the average carotid blood flow over 90 min. Control carotid blood flow in these groups averaged 23 ml/min. Average carotid blood flow over 90 min was reduced to less than 20% in vehicle-treated animals after electrical stimulation (Fig. 4). Aspirin, DuP 714, and rTAP caused a dose-dependent increase in average carotid blood flow over 90 min with ED₅₀ values of 56, 0.14, and 0.06 μmol/kg/h, respectively (Fig. 4). Values of time to occlusion (in min) for the vehicle and aspirin at 2, 6, 17, and 56 μmol/kg/h were 23 ± 2, 26 ± 4, 28 ± 5, 69 ± 9, and 88 ± 3, respectively; for the vehicle and DuP 714 at 0.06, 0.2, and 0.6 μmol/kg/h were 25 ± 3, 38 ± 8, 90 ± 0, and 90 ± 0, respectively; and for the vehicle and rTAP at 0.03 and 0.05 μmol/kg/h were 25 ± 3, 40 ± 6, and 75 ± 11, respectively. Significant increases in time to occlusion were observed for aspirin at 17 and 56 μmol/kg/h, for DuP 714 at 0.06 to 0.6 μmol/kg/h, and for rTAP at 0.05 μmol/kg/h (P < .05, compared with vehicle).

Figure 5 shows effects of vehicle and SK549 on carotid blood flow after electrical stimulation. Control carotid blood flow in these animals averaged 24 ml/min. After electrical stimulation, blood flow was gradually decreased and the artery was totally occluded in about 35 min in vehicle-treated animals. SK549 (0.04 to 1.5 μmol/kg/h i.v.) caused a dose-dependent increase in duration of the patency of the artery. At 0.45 and 1.5 μmol/kg/h SK549, there was no occlusion in all the animals up to 90 min. Values of time to occlusion (in min) for the vehicle and SK549 at 0.04, 0.09, 0.15, 0.45, and 1.5 μmol/kg/h were 25 ± 3, 34 ± 5, 78 ± 5, 86 ± 4, 90 ± 0, and 90 ± 0, respectively. SK549 caused significant increases in time to occlusion at 0.09 to 1.5 μmol/kg/h (P < .05, compared with vehicle). Figure 6 shows antithrombotic effects of SK549 expressed as average carotid blood flow over 90 min. Average blood flow over 90 min was decreased to 17 ± 2% of control level in vehicle-treated animals. SK549 caused a dose-dependent increase in average blood flow with an ED₅₀ of 0.12 μmol/kg/h i.v. At 1.5 μmol/kg/h i.v., SK549 maintained carotid blood flow at 87 ± 6% of control level for greater than 90 min. In addition, we observed a good correlation between the doses and the plasma concentrations reached after i.v. infusion of SK549 (Fig. 7, r = 0.96, P < .0001). The EC₅₀ for SK549 was estimated to be 97 nM.

**Blood Pressure and Heart Rate Effects of SK549.** Effects of the saline vehicle and SK549 on blood pressure and heart rate were evaluated in a separate group of animals. Compared with the vehicle (n = 4), SK549 at 1.5 μmol/kg/h i.v. (n = 4) did not change blood pressure significantly (76 ± 3 mm Hg for SK549 and 86 ± 7 mm Hg for vehicle) and heart rate significantly (182 ± 13 bpm for SK549 and 173 ± 9 bpm for vehicle).

**Ex Vivo Effects of SK549 on Platelet Aggregation and Coagulation Parameters.** At 1.5 μmol/kg/h i.v. (n = 4), SK549 did not alter the ex vivo platelet aggregation induced by either ADP or γ-thrombin (ADP, 51 ± 3% for the control and 54 ± 4% for SK549; or γ-thrombin, 67 ± 4% for the control and 68 ± 6% for SK549). Figure 8 shows ex vivo effects of SK549 and heparin on IXa.
SK549 is a potent and selective fXa inhibitor with a $K_i$ of 0.52 nM against human fXa (Wong et al., 2000). Compared with other well characterized small-molecule fXa inhibitors, SK549 is 58, 2.5, 13.5, and 2.5 times more potent in terms of $K_i$ than DX-9065a ($K_i = 30$ nM), YM-60828 ($K_i = 1.3$ nM, reported by Taniuchi et al., 1998), RPR120844 ($K_i = 7$ nM, reported by Leadley et al., 1999), and RPR208566 ($K_i = 1.31$ nM, reported by Heran et al., 2000), respectively. In addition, given intraduodenally and i.v. to rabbits, SK549 effectively prevented thrombus formation in a model of arteriovenous shunt thrombosis (Wong et al., 2000). In this study, we demonstrated clearly that SK549 given i.v. to rabbits is also a potent antithrombotic agent in a model of arterial thrombosis.

Our study is the first comparative evaluation of the dose-dependent antithrombotic effects of small-molecule fXa inhibitors such as SK549, the peptide fXa inhibitor rTAP, standard heparin, and the direct thrombin inhibitor DuP 714 in a rabbit model of arterial thrombosis. Although the fXa inhibitor YM-60828 has been evaluated in the rat ECAT model (Kawasaki et al., 1998), the antithrombotic effect of a small-molecule fXa inhibitor has not been previously reported in a similar ECAT model in rabbits. Furthermore, we believe that the rabbit is a better choice of animal model than the rat for
evaluating the antithrombogenic effect of small-molecule fXa inhibitors. Studies have reported that rabbit fXa, but not rat fXa, and human fXa have similar binding affinity to enzyme substrate and small-molecule inhibitors of fXa (Hara et al., 1995; Taniuchi et al., 1998).

We reasoned that the ECAT model, which mimics clinical arterial thrombosis, may be physiologically more relevant than the arteriovenous shunt thrombosis model. The former but not the latter model involves additional factors that are important for the mechanism of thrombus formation, such as high shear rate and endothelial injury (Badimon, 1997). In this study, we used external electrical stimulation to induce endothelial injury. Scanning electron microscopy confirmed endothelial injury at the site where the electrical stimulation was applied. The injured vessel was covered with platelets and fibrin. The platelet deposition is consistent with the findings of Badimon (1997) showing that the de-endothelialized vessel wall, which is exposed to blood at high shear rate, would induce platelet deposition on the exposed vessel. In addition, the tissue factor in the subendothelial extracellular matrix of the denuded vessel would activate blood coagulation cascade and contribute significantly to thrombin formation and fibrin deposition (Pawashe et al., 1994).

We observed that heparin is a weak antithrombogenic agent in the ECAT rabbit model. Previously, we showed that heparin at 64 U/kg/h completely inhibited the thrombus formation in the rabbit arteriovenous shunt model (Wong et al., 2000). However, in this study heparin at a higher dose of 100 U/kg/h, which increased APTT by greater than 6-fold, did not prevent arterial thrombosis in rabbits. Our finding is consistent with previous reports showing that heparin is a weak antithrombogenic agent for the treatment of arterial thrombosis in humans and animals (Schumacher et al., 1993; Kawasaki et al., 1998; Lockyer and Kambayashi, 1999; Heran et al., 2000; Hirsh and Bates, 2000). This may be related to the ineffective inhibition of clot-bound fXa and thrombin by the complex of antithrombin III with heparin (Teitel and Rosenberg, 1983; Weitz et al., 1990). Unlike heparin, the potencies of nonpeptide fXa inhibitors tested in the ECAT and arteriovenous shunt model were very similar in both models. A possible explanation is that nonpeptide fXa inhibitors, because of their small size, may penetrate and inhibit the clot-bound fXa better than the complex of antithrombin III with heparin (Hérault et al., 1997).

Our study also shows that aspirin is not a very effective antithrombotic agent in the ECAT rabbit model, which is consistent with other reports showing that aspirin is a poor antithrombotic agent in similar ECAT models in rats (Bernat et al., 1993; Schumacher et al., 1993). It is believed that aspirin at the high dose we studied is effective in blocking the formation of platelet-aggregating prostaglandins such as thromboxane A2. However, at the same time aspirin also blocks the production of platelet-inhibitory prostaglandins such as prostacyclin, which may account for its poor antithrombotic effect in arterial thrombosis models (Bernat et al., 1993; Schumacher et al., 1993; Lockyer and Kambayashi, 1999). The weak antithrombotic potency of aspirin may also be related to its lack of effects on blood coagulation with thrombin and fibrin formation, which play a role in arterial thrombosis.

We observed that DuP 714 is a potent antithrombotic agent in the ECAT rabbit model. A similar antithrombotic efficacy of DuP 714 has also been reported in a rabbit model of arteriovenous shunt thrombosis (Knabb et al., 1992). In the ECAT rabbit model, the dose of DuP 714 to achieve maximal antithrombotic effect (reflected by an increase in blood flow) resulted in a 4.8-fold prolongation in APTT. In contrast, the maximal antithrombotic dose of SK549 only prolonged APTT by 2.4-fold. It is not known how much systemic anticoagulation can be tolerated without bleeding complications during antithrombotic therapy. However, bleeding complications occurred in clinical trials with hirudin (a naturally occurring direct thrombin inhibitor) for the treatment of myocardial infarction (for references, see Kaiser, 1998). Whether a lower level of anticoagulation induced by fXa inhibitors may account for a reduced incidence of bleeding complications in patients remains to be determined.

SK549 is a potent antithrombotic agent in the ECAT model with an EC$_{50}$ of 97 nM, which is very close to the potency of 62 nM observed in the arteriovenous shunt thrombosis model (Wong et al., 2000). Although SK549 was as effective as rTAP in the arteriovenous shunt thrombosis rabbits (Wong et al., 2000), it appears that SK549 was more effective than rTAP in the ECAT rabbits. For instance, rTAP at 0.05 µmol/kg/h, which exerted an antithrombotic effect of 91% in the arteriovenous shunt thrombosis rabbits (Wong et al., 2000), produced an antithrombotic effect of 46% (reflected by an increase in blood flow) in the ECAT rabbits. On the other hand, SK549 at 1.5 µmol/kg/h, which exerted an antithrombotic effect of 91% in the arteriovenous shunt thrombosis rabbits (Wong et al., 2000), produced an antithrombotic effect of 87%, respectively, in the ECAT rabbits. The mechanism responsible for the increased effectiveness of SK549 in the ECAT rabbits compared with rTAP is not clear, but could be related to the slow binding kinetics of rTAP to fXa (Eisenberg et al., 1992).

To substantiate that the antithrombotic effect of benzamidine isoxazoline fXa inhibitors is due to the inhibition of fXa, the correlation between the inhibitory constants for the fXa, $K_i$, and the in vivo antithrombotic potencies, ED$_{50}$, of a series of benzamidine isoxazoline fXa inhibitors was determined. Our study shows a good correlation between $K_i$ and ED$_{50}$, supporting that inhibition of fXa is the primary mechanism of the antithrombotic effect of these nonpeptide fXa inhibitors. This is further substantiated by the finding that SK549
at antithrombotic doses selectively inhibited ex vivo Fxa but not thrombin activity.

It should be noted that SK549 at 1.5 μmol/kg/h does not alter blood pressure and heart rate, suggesting that the involvement of hemodynamic effect in its antithrombotic effect is not likely. In addition, the antithrombotic effect of SK549 may not be due to inhibition of platelet aggregation because SK549 at the maximal antithrombotic dose of 1.5 μmol/kg/h did not inhibit the ex vivo platelet aggregation induced by ADP or γ-thrombin.

Some studies have used the time to occlusion as an index of antithrombotic effect (Kawasaki et al., 1998; Heran et al., 2000), which, we believe, may overestimate the antithrombotic efficacies of the compounds. For instance, this study shows that aspirin and rTAP could produce high values of time to occlusion, but low-to-moderate levels of blood perfusion (as reflected by the blood flow). Thus, the time to occlusion has important limitations as an index of antithrombotic effect, which must be taken into account in the interpretation of antithrombotic effect of a test agent.

APTT is universally used to monitor the therapeutic level of heparin-induced anticoagulation (Kher et al., 1997; Bajaj and Joist, 1999). The dose of heparin that doubles the APTT is often taken as a measure of adequate heparin administration. In this study, although heparin at 100 U/kg/h i.v. increased APTT by greater than 6-fold, it had only a minimum antithrombotic effect in the ECAT rabbit model. Heparin at this dose also increased TT by greater than 7-fold and did not change PT. In contrast, the maximal antithrombotic effect of SK549 was associated with a 2.4-fold increase in APTT and less than 2-fold increase in PT. SK549 did not change TT, supporting that the antithrombotic effect of SK549 is not related to thrombin inhibition. Although APTT and PT are very useful for monitoring heparin and warfarin therapy, respectively, in the clinic (Kher et al., 1997; Bajaj and Joist, 1999), our study shows that these tests are not sensitive enough to monitor the antithrombotic effect of the Fxa inhibitor SK549. It appears that measuring anti-Fxa activity is a sensitive method for assessing Fxa inhibitors ex vivo.

In summary, our study shows that SK549 is a novel, potent, and effective antithrombotic agent in a rabbit model of arterial thrombosis. It is likely that SK549 exerts its antithrombotic effect through selective inhibition of Fxa. Thus, SK549 may be clinically useful for the prevention of arterial thrombosis.

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References


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