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Comparison of Thrombin Active Site and Exosite Inhibitors and Heparin in Experimental Models of Arterial and Venous Thrombosis and Bleeding

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ABSTRACT

Different pharmacological approaches to thrombin inhibition were compared for their effects on thrombosis and bleeding time in anesthetized rats. Thrombosis was induced in the carotid artery by transmural vessel injury and in the vena cava by partial blood flow stasis combined with mild endothelial disruption. Small mesenteric arteries were punctured with a hypodermic needle to measure the bleeding time. Dose-response relationships were determined with a thrombin active site inhibitor, N-methyl D-Phe-Pro-Arg-aldehyde (GYKI 14,766); a thrombin exosite inhibitor, succinyl-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-cyclohexylalanine-Gln (BMS 180,742); and heparin. BMS 180,742 interferes with fibrinogen binding to the thrombin exosite but, unlike GYKI 14,766, it does not block thrombin's catalytic site. The effects on thrombosis and bleeding time were correlated with *ex vivo* clotting times using the activated partial thromboplastin time for heparin and the thrombin time for GYKI 14,766 and BMS 180,742. Venous thrombosis was inhibited more than 90% by all three inhibitors at doses that either produced threshold increases or had no effect on bleeding and clotting times. Arterial thrombosis was inhibited 82% by GYKI 14,766 and 63% by heparin but it was not inhibited by BMS 180,742. These antithrombotic activities were accompanied by a maximal activated partial thromboplastin time increase and doubling of the bleeding time with heparin and a maximal thrombin time prolongation and 35% increase in bleeding time with GYKI 14,766. These results suggest that thrombin inhibitors, which act at the active site or exosite or through antithrombin III, are equally efficacious against venous thrombosis but active site inhibitors are the most effective against arterial thrombosis.

The central role of thrombin in blood coagulation and platelet activation has made it a prominent target for the development of drugs designed to limit hemostasis under pathological conditions (Okunomiya and Okamoto, 1992). Thrombin has a catalytic active site and a fibrinogen recognition exosite that are both involved in thrombin-mediated platelet activation and fibrin formation (Fenton *et al.*, 1991). The catalytic site is responsible for the serine protease activity of thrombin. The anionic-binding exosite is believed important for macromolecular substrate recognition and the orientation of substrate cleavage sites to thrombin's catalytic site. These binding sites are distinct from the exosite to which heparin binds and, therefore, they offer pharmacologists two distinct additional targets for developing thrombin inhibitors.

GYKI 14,766 is a synthetic peptide that inhibits the active site of thrombin in a reversible manner (Bagdy *et al.*, 1992a). The antithrombotic activity of GYKI 14,766 has been demonstrated in experimental rat models of arterial and venous thrombosis (Bagdy *et al.*, 1992b). BMS 180,742 is a synthetic peptide based on the carboxy terminus of hirudin and it inhibits substrate binding to thrombin's fibrinogen recognition exosite in a reversible manner. Unlike hirudin, BMS 180,742 does not have the additional effect of inhibiting thrombin's active site. BMS 180,742 has been shown to inhibit thrombin-induced plasma clotting and platelet activation; however, its antithrombotic activity is not known (Seiler *et al.*, 1991, 1992). Others found that different thrombin fibrinogen-binding exosite inhibitors are effective in models of venous thrombosis (Broersma *et al.*, 1991) but they appear less effective in the setting of arterial thrombosis (Kelly *et al.*, 1992). Although studies to date clearly demonstrated the antithrombotic efficacy of active site and exosite thrombin inhibitors, the activity of these inhibitors in venous and arterial thrombosis relative to their ability to increase bleeding has not been fully described.

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ABBREVIATIONS: APTT, activated partial thromboplastin time; CBF, carotid blood flow; GYKI 14,766, N-methyl D-Phe-Pro-Arg-aldehyde; BMS 180,742, succinyl-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-cyclohexylalanine-Gln; TT, thrombin time.

We characterized the dose-response relationships of GYKI 14,766, BMS 180,742 and heparin in the inhibition of arterial and venous thrombosis and as agents that increase small artery bleeding. These studies were performed with rats because of the large number of animals required to define all the separate dose-response relationships. Our arterial thrombosis model was modified from Kurz *et al.* (1990) and used vascular injury induced by $FeCl_2$ to produce platelet-rich and fibrin-rich thrombi. We found this model to be insensitive to a wide range of aspirin doses (Schumacher *et al.* 1993). The venous thrombosis model was modified from that of Millet *et al.* (1987). It used a combination of blood flow stasis with mild endothelial disruption to induce formation of a whole blood clot in the vena cava. The bleeding time was measured in small mesenteric arteries by the method of Zawilska *et al.* (1982). We also correlated these biological activities with selected clotting times determined *ex vivo*.

Methods

Arterial thrombosis. Male Sprague-Dawley rats (weight range, 350–450 g) were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Their jugular veins were cannulated with polyethylene-20 or -50 tubing for drug administration or anesthetic supplementation and their tracheas were intubated to ensure airway patency. The right carotid artery was exposed and a piece of Parafilm M (American Can Co., Greenwich, CT) was placed under the vessel. An electromagnetic flow probe (0.95- or 1.0-mm lumen) was placed on the artery and attached to a model MDL 1401 flowmeter (Skalar, Delft, Netherlands). When CBF had stabilized, a 2 × 5 mm strip of filter paper was saturated in a 50% solution of $FeCl_2$ and placed on top of the vessel downstream from the flow probe. The filter paper was removed after 10 min. Drug (GYKI 14,766, BMS 180,742 or heparin) or vehicle infusions were begun 15 min before the $FeCl_2$ application. The carotid artery was removed on occlusion or 60 min after the filter paper application if patency was maintained. The vessel was opened lengthwise under a stereomicroscope and the white thrombus was removed, excluding any loosely attached clotted blood. The wet weight of the thrombus was determined immediately on a Sartorius R-160P balance (Brinkmann Instruments, Westbury, NY). The CBF was monitored continuously and the total CBF was calculated by computer-assisted planimetry. To obtain the average CBF, the total CBF was normalized to the percent of base-line (0 min) flow over 60 min.

Venous thrombosis. Male Sprague-Dawley rats (350–450 g) were anesthetized. Their tracheas were intubated and their jugular veins were catheterized as described earlier. The vena cava was isolated by a midline abdominal incision. A caval sac was produced by tying a ligature around a 26-gauge needle just distal to the renal veins and applying a microaneurysm clamp just proximal to the bifurcation of the femoral veins. A separate 26-gauge hypodermic needle was inserted into the inferior portion of the venous sac and hypotonic saline (0.225%) was infused at 10 ml/min for 15 sec. The hypodermic needle was removed from the caval sac after the saline infusion and the hole was sealed with cyanoacrylate cement. The proximal needle was then freed from the ligature to leave a fixed nonocclusive stenosis and the distal vascular clamp was removed. After 20 min, the sac was redefined with vascular clamps, removed from the rat and slit open lengthwise. The exposed thrombus was extracted and its wet and dry weights were determined. Treatments (GYKI 14,766, BMS 180,742, heparin or vehicle) were begun 15 min before sac construction.

Mesenteric artery bleeding time. Male Sprague-Dawley rats (350–450 g) were anesthetized. Their tracheas were intubated and their jugular veins were catheterized as described earlier. The jejunum was isolated through a midline laparotomy and it was exteriorized. Arteries branching perpendicular from the mesenteric artery over the surface of the jejunum were visualized under a stereomicroscope. Individual arteries were punctured with a 30-gauge hypodermic needle while superfusing the intestinal surface with Ringer's solution warmed to 37°C. The bleeding time was determined by cessation of the free-flowing blood from the puncture site. The bleeding times were measured in four to six vessels before (control) and 15 min after GYKI 14,766, BMS 180,742, heparin or vehicle infusion was begun. The control and post-treatment bleeding times from each rat were averaged to a single control and single post-treatment values.

Clotting times. In some experiments, blood was withdrawn into a 1/10th final volume of 3.8% sodium citrate after 35 to 60 min of drug infusion. Blood samples were centrifuged for 2 min in an Eppendorf 5412 microcentrifuge (Brinkman Instruments). Plasma was withdrawn, frozen immediately at −70°C and rethawed once for clotting time determinations. The APTT was measured on a Fibrometer using the procedure for Actin FSL APTT Reagent (Baxter Healthcare, Miami, FL). The TT was measured optically using an Electra 700 (Medical Laboratory Automation, Pleasantville, NY) by adding 10 U/ml of human α-thrombin (Sigma, St. Louis, MO) to plasma diluted 1:1 in Owren's Veronal buffer (Baxter Healthcare). All clotting reactions were performed at 37°C.

Treatment protocols. GYKI 14,766 (Gedeon Richter, Budapest, Hungary; molecular weight = 517g/mol), BMS 180,742 (Bristol-Myers Squibb, Princeton, NJ; molecular weight = 1404g/mol), heparin (derived from beef lung; Upjohn, Kalamazoo, MI) and vehicle were administered in i.v. doses as a 1-ml bolus injection plus a 25-μl/min continuous infusion. The total infusion times were 35 min for venous thrombosis and bleeding time and 75 min for arterial thrombosis. The doses and the numbers of animals per dose are indicated in figures 1 to 6. The vehicle was saline for the heparin group in arterial thrombosis and a 10% solution of ethanol plus 0.2% sodium carbonate in all other experiments.

Data analyses. Drug effects were determined by analysis of variance using a repeated-measures design when possible. In the arterial thrombosis model, base-line CBF was added as a covariate in the total CBF analysis. The percent changes in bleeding time were compared between drug and vehicle groups by analysis of covariance with the average control bleeding time as the covariate. This was the most sensitive method of detecting drug effects on bleeding time. Contrasts were used to compare mean differences among treatments. Computations were performed on a Macintosh computer using Systat software (Apple, Evanston, IL). All data are presented as the mean ± S.E.M. The null hypothesis was rejected for $P < .05$.

Results

Arterial thrombosis. The three separate vehicle groups did not differ significantly in thrombus weights or base-line CBF. Thrombus weights in vehicle-treated rats averaged 4.9 ± 0.5, 4.5 ± 0.5 and 3.8 ± 0.3 mg in the GYKI 14,766 ($n = 11$), BMS 180,742 ($n = 8$) and heparin ($n = 8$) studies, respectively. Overall, occlusive thrombosis was observed in 24 of 27 vehicle-treated rats. Of those rats that had occlusions, the average time to occlusion was 20 ± 1 min (range, 15–25 min). GYKI 14,766 and heparin each produced a dose-dependent inhibition of thrombus weight with maximum decreases of 82% and 63%, respectively (figs. 1 and 2). At the highest doses tested, GYKI 14,766 (3 mg/kg and 150 $\mu g\ kg^{-1}\ min^{-1}$) and heparin (300 U/kg and 10 $U\ kg^{-1}\ min^{-1}$) maintained vessel patency in all animals. In GYKI 14,766-treated rats, CBF did not decrease and was maintained at 98 ± 14% of the control level for the duration of the experiment. Unlike GYKI 14,766 and heparin, BMS 180,742 did not reduce thrombus weight or improve CBF during thrombus formation at doses up to 5 mg/kg and 250 $\mu g\ kg^{-1}\ min^{-1}$ (fig. 3).

The effects of GYKI 14,766, heparin and BMS 180,742 on *ex vivo* clotting times relative to their antithrombotic activities

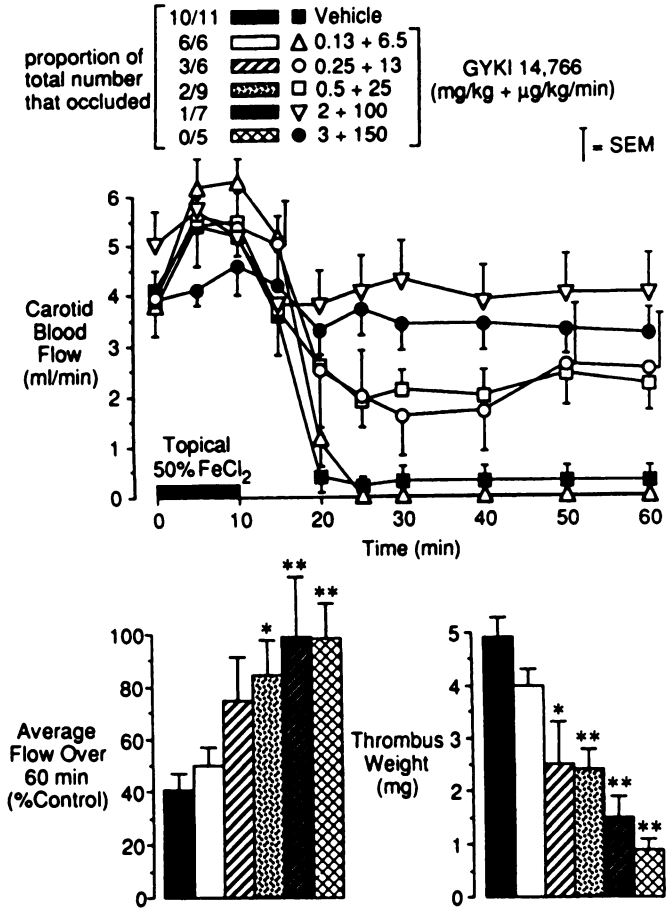

Fig. 1. The effects of the thrombin active site inhibitor, GYKI 14,766, on CBF during thrombosis and on arterial thrombus weight were determined in anesthetized rats. The following differences were detected by analysis of variance and contrasts: *P < .05 and **P < .01, respectively, compared with vehicle. The total amounts of drug administered in the order of increasing dose were: 0.6, 1.2, 2.4, 9.5 and 14.3 mg/kg.

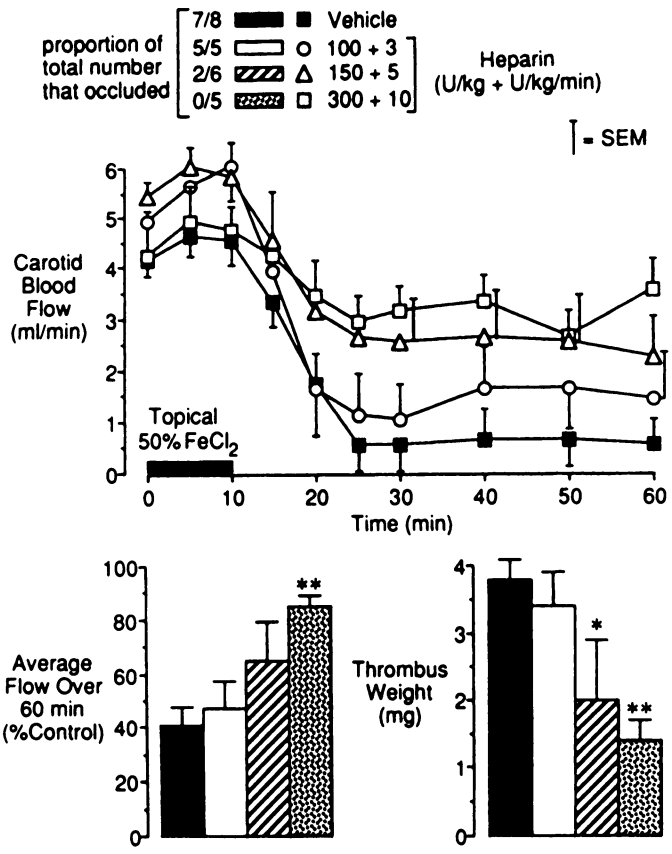

Fig. 2. The effects of heparin on CBF during thrombosis and on arterial thrombus weight were determined in anesthetized rats. The following differences were detected by analysis of variance and contrasts: *P < .05 and **P < .01, respectively, compared with vehicle. The total amounts of drug administered in the order of increasing dose were: 325, 525 and 1050 U/kg.

were variable. Heparin given at a dose of 150 U/kg and 5 U kg^{-1} min^{-1} caused a threshold 49% inhibition of thrombosis (fig. 2), whereas a maximal increase in APTT (> 100 sec) was achieved at a lower heparin dose of 100 U/kg and 3 U kg^{-1} min^{-1} (fig. 4). By contrast, the 0.25 mg/kg and 25 μg kg^{-1} min^{-1} dose of GYKI 14,766 produced 50% inhibition of thrombus formation and produced only a threshold prolongation of TT (fig. 4). The highest dose of BMS 180,742 (5 mg/kg and 250 μg kg^{-1} min^{-1}) had less of an effect on TT than did a high dose of GYKI 14,766 (2 mg/kg and 100 μg kg^{-1} min^{-1}). Nevertheless, this dose of BMS 180,742 prolonged TT to a greater extent than did an effective antithrombotic dose of GYKI 14,766 (0.25 mg/kg and 25 μg kg^{-1} min^{-1} (fig. 4).

Venous thrombosis. GYKI 14,766, BMS 180,742 and heparin all abolished vena cava thrombus formation in a dose-dependent manner (fig. 5). All three drugs achieved threshold antithrombotic activity at doses that did not significantly affect clotting times (fig. 4). Although only data on wet thrombus weights are shown, the same significant differences were obtained when dry weights were analyzed.

Bleeding time. Bleeding times did not differ before (82.0 ± 6.7 sec) and after (81.5 ± 7.0 sec) vehicle treatment (n = 10). Mesenteric bleeding times were prolonged by GYKI 14,766, BMS 180,742 and heparin but the dose of each drug required for this effect exceeded the threshold dose necessary for inhibition of caval thrombosis (fig. 6). Maximal increases in bleeding time were about 100% for heparin and in the range of 30% to 40% for GYKI 14,766 and BMS 180,742. Rebleeding from control sites was not observed after any of the drug treatments.

Discussion

This study is novel in its description of the dose-dependent effects of diverse thrombin inhibitors in arterial and venous thrombosis and in its correlation of these activities with the effects on bleeding and *ex vivo* clotting times. Arterial thrombosis was inhibited by the thrombin active site inhibitor GYKI 14,766 and heparin but not by the thrombin exosite inhibitor BMS 180,742. This was expected based on previous studies that demonstrated the antithrombotic activity of heparin (Hladovec, 1973; Philp *et al.*, 1978; Schumacher *et al.*, 1992) and active site thrombin inhibitors (Bagdy *et al.*, 1992b) in rat models of arterial thrombosis. Aside from establishing efficacy, it is important to know the dose-response relationship between antithrombotic activity and other therapeutically relevant parameters. *Ex vivo* clotting times are commonly used to select optimal therapeutic doses of anticoagulants. Clinical use of heparin usually targets about a 2-fold increase in APTT. We found that heparin produced approximately a 3-fold increase in APTT at a dose that was only one-tenth of that required for threshold inhibition of arterial thrombosis. In fact, the maximal 63% inhibition of arterial thrombosis obtained with heparin

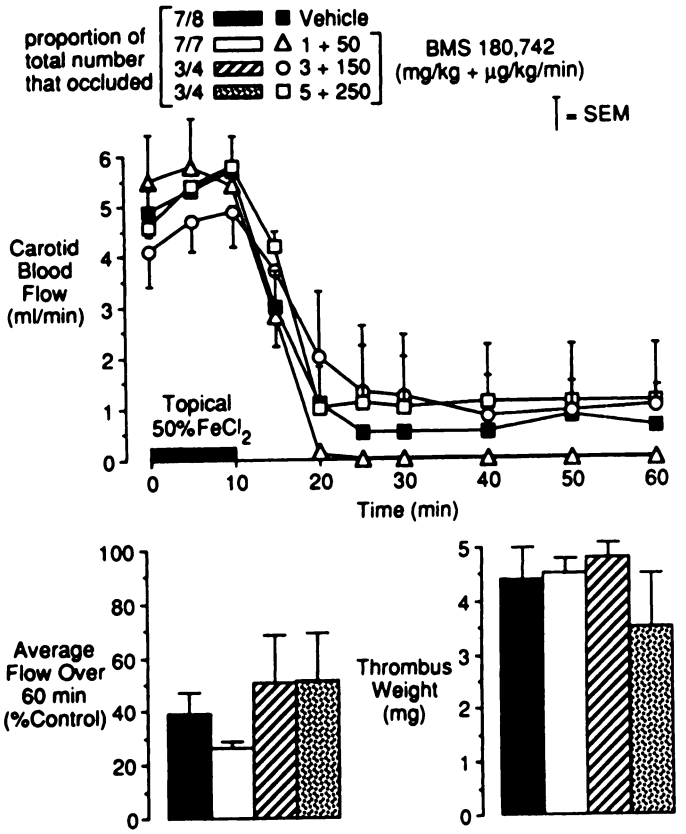

Fig. 3. The effects of the thrombin exosite site inhibitor, BMS 180,742, on CBF during thrombosis and arterial thrombus weight were determined in anesthetized rats. There were no differences detected in the average blood flow and thrombus weight between vehicle- and BMS 180,742-treated rats by analysis of variance and contrasts. The total amounts of drug administered in the order of increasing dose were: 4.8, 14.3 and 23.8 mg/kg.

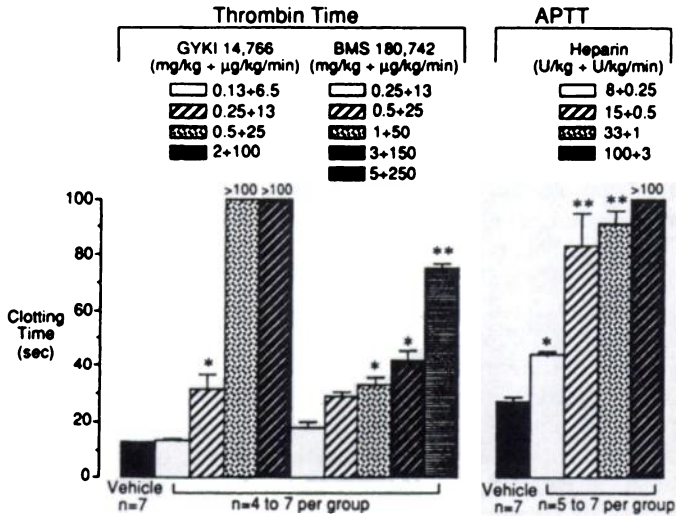

Fig. 4. The effects of a thrombin active site inhibitor (GYKI 14,766), a thrombin exosite inhibitor (BMS 180,742) and heparin on *ex vivo* clotting times were determined in anesthetized rats. The maximal clotting time was 100 sec for both the TT and APTT. The following differences were detected by analysis of variance and contrasts: *P < .05 and **P < .01, respectively, compared with each vehicle. Bars = S.E.M.

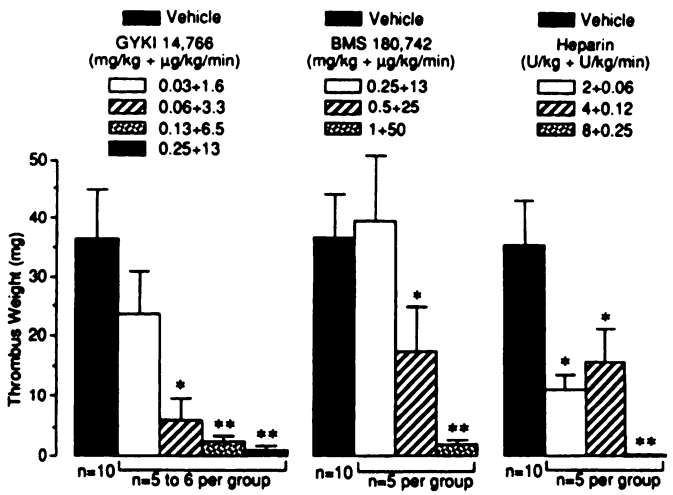

Fig. 5. The effects of a thrombin active site inhibitor (GYKI 14,766), a thrombin exosite inhibitor (BMS 180,742) and heparin on vena cava thrombosis were determined in anesthetized rats. The following differences were detected by analysis of variance and contrasts: *P < .05 and **P < .01, respectively, compared with each vehicle. Bars = S.E.M.

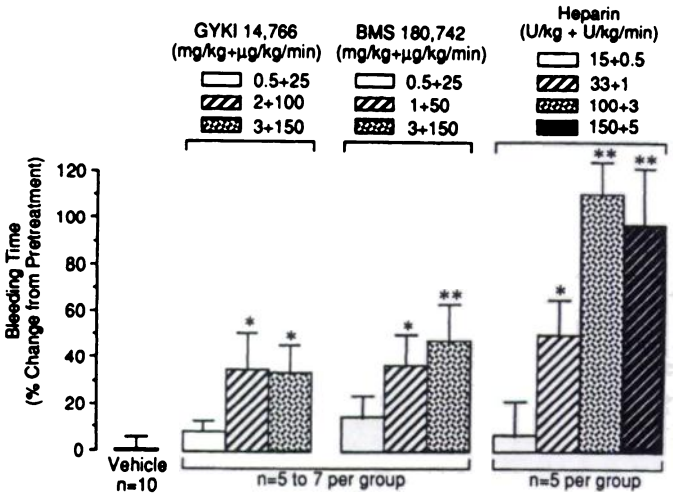

Fig. 6. The effects of a thrombin active site inhibitor (GYKI 14,766), a thrombin exosite inhibitor (BMS 180,742) and heparin on mesenteric arterial bleeding time were determined in anesthetized rats. The following differences were detected by analysis of variance and contrasts: *P < .05 and **P < .01, respectively, compared with vehicle. Pretreatment bleeding times averaged 83 ± 16 sec overall (± S.D., n = 63) and did not differ significantly between any of the drug-treated and vehicle groups. Bars = S.E.M.

maximally prolonged APTT. For this reason, the observed antithrombotic activity of heparin in arterial thrombosis is not clinically relevant. The clinical efficacy of heparin during coronary artery thrombolysis may relate more to its effectiveness in venous thrombosis. In experimental studies of thrombolysis performed without anticoagulants, intermittent stasis of blood flow through a stenotic coronary artery during initial reflow resulted in the formation of large venous-like clots downstream from the partially lysed thrombus (Schumacher *et al.*, 1985). Explanations for the limited potency of heparin against arterial thrombosis include the insensitivity of clot-bound thrombin to heparin (Weitz *et al.*, 1990) and inhibition of heparin by a fibrin monomer (Hogg and Jackson, 1989).

At present, there is no universally recognized clinical clotting assay for direct thrombin inhibitors, although in preliminary experiments, we found TT to be more sensitive to this class of

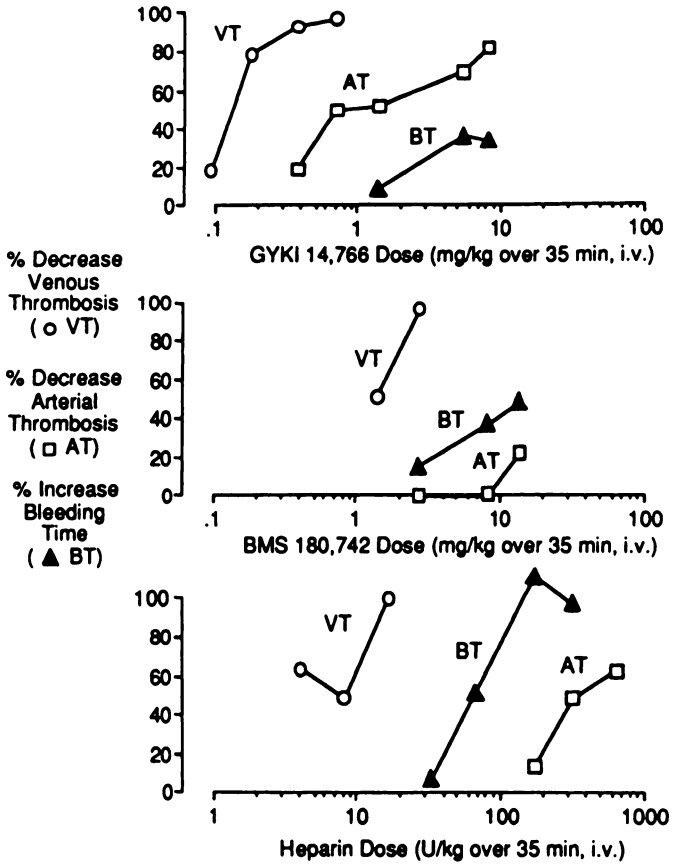

Fig. 7. The dose-dependent effects of a thrombin active site inhibitor (GYKI 14,766), a thrombin exosite inhibitor (BMS 180,742) and heparin on arterial thrombosis (AT), venous thrombosis (VT) and bleeding time (BT) are summarized. The data are the mean values obtained from figures 1, 2, 3, 5 and 6. The doses were calculated as the total drug administered over 35 min. This corresponds to the amount of each drug that was infused either at the end of the post-treatment BT measurements, at the time of clot removal in the VT model or at the average occlusion time in the AT model for vehicle-treated rats.

drugs than APTT or prothrombin time. The 2.5-fold increase in TT produced by GYKI 14,766 correlated with a 50% reduction in arterial thrombus weight. However, the highest dose of GYKI 14,766 that maximally inhibited arterial thrombosis also elicited maximal increases in TT.

BMS 180,742 did not affect arterial thrombosis at doses that increased the TT up to 6-fold, which was less than the effect of antithrombotic doses of GYKI 14,766 on TT. We found that the concentration of BMS 180,742 required to double TT was the same for human (0.66 μM) and rat (0.5 μM) thrombin (Seiler *et al.*, 1991, and unpublished data). This characterized the rat as being adequate for testing this compound. Our results corroborate those of Kelly *et al.* (1992), *i.e.*, targeting thrombin's active site produced greater activity in models of arterial thrombosis than did targeting its fibrinogen-binding exosite. There may be several reasons for the lack of efficacy of BMS 180,742 against arterial thrombosis. Inhibition by BMS 180,742 of thrombin-induced platelet activation *in vitro* can be completely reversed by increasing the thrombin concentration to ~10 U/ml (Seiler *et al.*, 1991), which is well within the range achieved during the clotting of whole blood (Liu *et al.*, 1979). Our current understanding is that α-thrombin binds to a platelet receptor through its exosite and cleaves the receptor's N-terminus to produce a tethered-ligand, which then activates the platelet (Ishii *et al.*, 1993). However, if the thrombin concentration is high enough, this exosite interaction may not be necessary because platelet thrombin receptors can be activated by trypsin and γ-thrombin, both of which do not have exosite-binding domains. Thrombin production *in vivo* may therefore override inhibition of its fibrinogen-binding exosite. Another consideration is thrombin's ability to amplify its own production by activating factors V and VIII. These reactions are inhibited by active site binding but not by fibrinogen exosite-binding inhibitors (Ofosu *et al.*, 1992). Furthermore, the inhibition of factors V and VIII by protein C is impaired by thrombin exosite inhibitors that prevent the binding of thrombin to thrombomodulin which is required for protein C activation (Tsiang *et al.*, 1990).

The therapeutic range of thrombin inhibitors is likely to be limited by bleeding complications. The bleeding that is the most serious and difficult to treat occurs internally and this is not predicted well by skin template bleeding times (Rodgers and Levin, 1990). Mesenteric artery bleeding time is a highly reproducible index of internal bleeding in rats and it is sensitive to cyclooxygenase inhibition (Juan and Sametz, 1989). Drug effects on hemostasis in small *versus* large arteries may differ because of differences in blood flow and the relative degree of contact between endothelial cells and blood, which is greater in small vessels. Doses of heparin necessary to inhibit arterial thrombosis doubled the mesenteric bleeding time and BMS 180,742 increased bleeding without inhibiting arterial thrombosis. By contrast, GYKI 14,766 significantly reduced both venous and arterial thrombosis without affecting bleeding and, at optimal antithrombotic doses, it had less of an effect on bleeding than did heparin. The dose-response relationship of each thrombin inhibitor defined in models of arterial and venous thrombosis and bleeding time is shown in figure 7. The superior selectivity of GYKI 14,766 for arterial thrombosis is clearly evident in this figure. Jackson *et al.* (1992) obtained a qualitatively similar profile of activities when comparing heparin and GYKI 14,766 in canine models of arterial thrombosis and bleeding.

Many experimental models of venous thrombosis use artificial surfaces or procoagulant substances such as thromboplastin to initiate coagulation. The model of Millet *et al.* (1987) relies on endogenous mediators rather than exogenous procoagulant substances and it is extremely sensitive to heparin (Schumacher and Heran, 1989). All the thrombin inhibitors had equal and maximal efficacy in this model of venous thrombosis. Each inhibitor achieved this activity with little or no effect on the bleeding time (fig. 7). The threshold doses used to decrease caval thrombosis and cause bleeding prolongation were separated by a factor of ~30 for GYKI 14,766 and ~5 for both BMS 180,742 and heparin.

Overall, these experiments suggest that active site thrombin inhibitors are both more effective in arterial thrombosis and less prone to increase bleeding than are fibrinogen-binding exosite and antithrombin III-dependent thrombin inhibitors. The ability of the active site inhibitor to inactivate thrombin in large vessels in a manner that is less prone to regulatory mechanisms or increased thrombin generation, which leads to platelet activation, may explain its greater efficacy in arterial thrombosis. Differences among the three types of thrombin inhibitors in their effects on bleeding time are more difficult to reconcile. However, in general, we found that broad-spectrum antiplatelet drugs increase mesenteric bleeding and cause re-

bleeding to a greater extent than do thrombin inhibitors (unpublished studies). All three methods of thrombin inhibition were equally efficacious against venous thrombosis, which suggests that less thrombin inhibition is required for venous compared with arterial thrombosis.

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References

BAGDY, D., BARABÁS, E., BAJUSZ, S. AND SZÉLL, E.: *In vitro* inhibition of blood coagulation by tripeptide aldehydes—A retrospective screening study focused on the stable D-MePhe-Pro-Arg-H · H_2SO_4. Thromb. Haemostasis **67:** 325–330, 1992a.

BAGDY, D., SZABÓ, G., BARABÁS, E. AND BAJUSZ, S.: Inhibition by D-MePhe-Pro-Arg-H (GYKI 14,766) of thrombus growth in experimental models of thrombosis. Thromb. Haemostasis **68:** 125–129, 1992b.

BROERSMA, R. J., KUTCHER, L. W., HEMINGER, E. F., KRSTENANSKY, J. L. AND MARSHALL, F. N.: Antithrombotic activity of a novel C-terminal hirudin analog in experimental animals. Thromb. Haemostasis **65:** 377–381, 1991.

FENTON, J. W., II, OFOSU, F. A., MOON, D. G. AND MARAGANORE, J. M.: Thrombin structure and function: Why thrombin is the primary target for antithrombotics. Blood Coag. Fibrinolysis **2:** 69–75, 1991.

HLADOVEC, J.: Experimental arterial thrombosis in rats with continuous registration. Thromb. Diath. Heamorrh. **29:** 407–410, 1973.

HOGG, P. J. AND JACKSON, C. M.: Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: Implication for heparin efficacy. Proc. Natl. Acad. Sci. U. S. A. **86:** 1476–1484, 1989.

ISHII, K., HEIN, L., KOBILKA, B. AND COUGHLIN, S. R.: Kinetics of thrombin receptor cleavage on intact cells. J. Biol. Chem. **268:** 9780–9786, 1993.

JACKSON, C. V., CROWE, G., FRANK, J. D., WILSON, H. C., COFFMAN, W. J., UTTERBACK, B. G., JAKUBOWSKI, J. A. AND SMITH, G. F.: Pharmacological assessment of the antithrombotic activity of the peptide thrombin inhibitor, d-methyl-phenylalanyl-prolyl-arginal (GYKI 14,766), in a canine model of coronary artery thrombosis. J. Pharmacol. Exp. Ther. **261:** 546–552, 1992.

JUAN, H. AND SAMETZ, W.: Fish oil diet rich in eicosapentaenoic acid increases bleeding time in the rat by interaction with sympathetic transmitters. Agents Action **28:** 130–136, 1989.

KELLY, A. B., MARAGANORE, J. M., BOURDON, P., HANSON, S. R. AND HARKER, L. R.: Antithrombotic effects of synthetic peptides retargeting various functional domains of thrombin. Proc. Natl. Acad. Sci. U. S. A. **89:** 6040–6044, 1992.

KURZ, K. D., MAIN, B. W. AND SANDUSKY, G. I.: Rat model of arterial thrombosis induced by ferric chloride. Thromb. Res. **60:** 269–280, 1990.

LIU, C. Y., NOSSEL, H. L. AND KAPLAN, K. L.: The binding of thrombin to fibrin. J. Biol. Chem. **254:** 10421–10425, 1979.

MILLET, J., THEVENIAUX, J. AND PASCAL, M.: A new experimental model of venous thrombosis in rats involving partial stasis and slight endothelium alterations. Thromb. Res. **45:** 123–133, 1987.

OFOSU, F. A., FENTON, J. W., II, MARAGANORE, J., BLAJCHMAN, M. A., YANG, X., SMITH, L., ANVARI, N., BUCHANAN, M. R. AND HIRSH, J.: Inhibition of the amplification reactions of blood coagulation by site-specific inhibitors of α-thrombin. Biochem. J. **283:** 893–897, 1992.

OKUNOMIYA, A. H. AND OKAMOTO, S.: A strategy for a rational approach to designing synthetic selective thrombin inhibitors. Semin. Thromb. Hemostasis **18:** 135–149, 1992.

PHILP, R. B., FRANCEY, I. AND WARREN, B. A.: Comparison of antithrombotic activity of heparin, ASA, sulfinpyrazone and VK744 in a rat model of arterial thrombosis. Haemostasis **7:** 282–293, 1978.

RODGERS, R. P. C. AND LEVIN, J.: A critical reappraisal of the bleeding time. Semin. Thromb. Hemostasis **16:** 1–20, 1990.

SCHUMACHER, W. A. AND HERAN, C. L.: Effect of thromboxane receptor antagonists on venous thrombosis in rats. J. Pharmacol. Exp. Ther. **248:** 1109–1115, 1989.

SCHUMACHER, W. A., HERAN, C. H., STEINBACHER, T. E., DURHAM, S. K., YOUSSEF, S., MEGILL, J. R. AND OGLETREE, M. L.: Superior activity of a thromboxane receptor antagonist compared to aspirin in experimental arterial and venous thrombosis. J. Cardiovasc. Pharmacol. **22:** 526–533, 1993.

SCHUMACHER, W. A., HERAN, C. H., STEINBACHER, T. E., MEGILL, J. R., BIRD, J. E., GIANCARLI, M. R. AND DURHAM, S. K.: Thrombin inhibition compared with other antithrombotic drugs in rats. Thromb. Res. **68:** 157–166, 1992.

SCHUMACHER, W. A., LEE, E. C. AND LUCCHESI, B. R.: Augmentation of streptokinase-induced thrombolysis by heparin and prostacyclin. J. Cardiovasc. Pharmacol. **7:** 739–746, 1985.

SEILER, S. M., GOLDENBERG, H. J., MICHEL, I. M., HUNT, J. T. AND ZAVOICO, G. B.: Multiple pathways of thrombin-induced platelet activation differentiated by desensitization and a thrombin exosite inhibitor. Biochem. Biophys. Res. Commun. **181:** 636–643, 1991.

SEILER, S. M., MICHEL, I. M. AND FENTON, J. W., II: Involvement of the "tethered-ligand" receptor in thrombin inhibition of platelet adenylate cyclase. Biochem. Biophys. Res. Commun. **182:** 1296–1302, 1992.

TSIANG, M., LENTZ, S. R., DITTMAN, W. A., WEN, D., SCARPATI, E. M. AND SADLER, J. E.: Equilibrium binding of thrombin to recombinant human thrombomodulin: Effect of hirudin, fibrinogen, factor Va, and peptide analogues. Biochemistry **29:** 10602–10612, 1990.

WEITZ, J. I., HUDOBA, M., MASSEL, D., MARAGANORE, J. AND HIRSH, J.: Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. J. Clin. Invest. **86:** 385–391, 1990.

ZAWILSKA, K. M., BORN, G. V. R. AND BEGENT, N. A.: Effect of ADP-utilizing enzymes on the arterial bleeding time in rats and rabbits. Br. J. Haematol. **50:** 317–325, 1982.

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