Chapter 2

In Vivo Models for the Evaluation of Antithrombotics and Thrombolitics

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Abstract

The development and application of animal models of thrombosis have played a crucial role in the discovery and validation of novel drug targets and the selection of new agents for clinical evaluation, and have informed dosing and safety information for clinical trials. These models also provide valuable information about the mechanisms of action/interaction of new antithrombotic agents. Small and large animal models of thrombosis and their role in the discovery and development of novel agents are described. Methods and major issues regarding the use of animal models of thrombosis, such as positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species specificity, and pharmacokinetics, are highlighted. Finally, the use of genetic models of thrombosis/hemostasis and how these models have aided in the development of therapies that are presently being evaluated clinically are presented.

Key words: Animal models, coronary thrombosis, antithrombotic agents, pharmacodynamics, Folts, Wessler, thrombocytopenia, genetic models.

1. Introduction

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. Three factors responsible for thrombogenesis are proposed including obstruction of blood flow, changes in the properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two, or all three factors of Virchow’s triad. Therefore, they differ with respect to the prothrombotic challenge, i.e., stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, laser-light), insertion of foreign
surface, or injection of a prothrombotic factor, as well as vessel type and animal species.

Roughly, two types of models can be differentiated (1): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor resulting in fibrin-rich “red” venous type thrombi and (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis resulting in platelet-rich “white” mural thrombi. This distinction is not strict because platelets and the coagulation system influence each other. Drugs that prevent fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in human, namely atherosclerosis or thrombophilias, are not addressed in the models. Thus, any model is limited with regard to clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. Despite these limitations, animal models predict the clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a recent review by Leadley et al. (2). The clinical usefulness of an antithrombotic drug is determined in part by safety/efficacy ratio with respect to bleeding risk. Assessment of this parameter of the hemostatic system should therefore be included in the models if possible.

The development of antithrombotic agents requires preclinical assessment of the biochemical and pharmacologic effects of these drugs. It is important to note that second- and third-generation antithrombotic drugs are devoid of in vitro anticoagulant effects, yet in vivo, by virtue of endogenous interactions, these drugs produce potent antithrombotic actions. The initial belief that an antithrombotic drug must exhibit in vitro anticoagulant activity is no longer valid. This important scientific observation has been possible only because of the availability of animal models.

Several animal models utilizing species such as rats, rabbits, dogs, pigs, and monkeys have been made available for routine use. Other animal species such as the hamster, mouse, cat, and guinea pig have also been used. Species variations are an important consideration in selecting a model and interpreting the results, as these variations can result in different antithrombotic effects. Rats and rabbits are the most commonly used species in which both arterial and venous thromboses have been investigated. Both pharmacologic and mechanical means have been used to produce a thrombogenic effect in these models. Both rat and rabbit models for studying bleeding effects of drugs have also been developed. The rabbit ear blood loss model is most commonly used to test the hemorrhagic effect of drugs. The rat tail bleeding models have also been utilized for the study of several antithrombotic drugs.

These animal models have been well established and can be used for the development of antithrombotic drugs. It is also pos-
sible to use the standardized bleeding and thrombosis models to predict the safety and efficacy of drugs. Thus, in addition to the evaluation of in vitro potency, the endogenous effects of antithrombotic drugs can be investigated. Such standardized methods can be recommended for inclusion in pharmacopoeial screening procedures. Numerous models have now been developed to mimic a variety of clinical conditions where antiplatelet and antithrombotic drugs are used, including myocardial infarction, stroke, cardiopulmonary bypass, trauma, peripheral vascular diseases, and restenosis. While dog and primate models are relatively expensive, they have also provided useful information on the pharmacokinetics and pharmacodynamics of antithrombotic drugs. The primate models in particular have been extremely useful, as the hemostatic pathways in these species are comparable to those in humans. The development of such agents as the specific glycoprotein IIb/IIIa inhibitor antibodies relies largely on these models. These models are of pivotal value in the development of antithrombotic drugs and provide extremely useful data on the safety and efficacy of new drugs developed for human use.

In most animal models of thrombosis, healthy animals are challenged with thrombogenic (pathophysiologic) stimuli and/or physical stimuli to produce thrombotic or occlusive conditions. These models are useful for the screening of antithrombotic drugs.

1.1. Stasis Thrombosis Model

Since its introduction by Wessler in 1959 (3), the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic agents. This model has also been adapted for use in rats (4). In the stasis thrombosis model, a hypercoagulable state is mimicked by the administration of one of a number of thrombogenic challenges, including human serum (5), thromboplastin (6), activated prothrombin complex concentrates (7), factor Xa (8) and recombinant relipidated tissue factor (9). Administration of such agents produces a hypercoagulable state. Diminution of blood flow achieved by ligating the ends of vessel segments serves to augment the prothrombotic environment. The thrombogenic environment produced in this model simulates venous thrombosis where both the blood flow and the activation of coagulation play a role in the development of a thrombus.

1.2. Models Based on Vessel Wall Damage

The formation of a thrombus is not solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a non-thrombogenic surface over which the blood flows. Disruption of the endothelium exposes sub-endothelial tissue factor and collagen, which activate the coagulation and platelet aggregation processes, respectively. Endothelial damage can be induced experimentally by physical means.
(clamping, catheter), chemical means (fluorescein isothiocyanate, Rose Bengal, ferrous chloride), thermal injury, or electrolytic injury.

Each setting in the design of an animal model can answer specific question in relation to certain thrombotic disorders in human. However, the ultimate model of human thrombosis is in human.

1.3. Issues to be Taken in Consideration in Evaluating Antithrombotics

1.3.1. Effect on Hemostasis

Antithrombotic and anticoagulant drugs are effective in the control of thrombogenesis at various levels. These drugs are also capable of producing hemorrhagic effects that cannot be predicted using in vitro testing methods. The bleeding effects of a drug may be direct or indirect; hence assessing efficacy/safety ratios could be a useful parameter.

*Single versus repeated exposure*. Repeated administration of drugs can result in a cumulative response that may alter the pharmacokinetic and pharmacodynamic indices of a given agent. It is only through the use of animal models that such information can be generated. Furthermore, since antithrombotic drugs represent a diverse class of agents, their interactions with physiologically active endogenous proteins can only be studied using animal models.

1.3.2. Choice of Species

Species variation plays an important role in thrombotic, hemostatic, and hemorrhagic responses. While there is no set formula to determine the relevance of the results obtained with animal models to man, the use of animal models can provide valuable information on the relative potency of drugs, their bioavailability after various routes of administration, and their pharmacokinetic behavior. Specific studies have provided data on the species relevance of the responses in different animal models to the projected human responses. Thus, the use of animal models in the evaluation of different drugs can provide useful data to compare different drugs within a class. However, caution must be exercised in extrapolating such results to the human clinical condition.

1.3.3. Selection of Animal Model

The selection of animal model for the evaluation of antithrombotic effects depends on several factors. The interaction of a given drug with the blood and vascular components and its metabolic transformation are important considerations. Thus, ex vivo analysis of blood along with the other endpoints can provide useful information on the effects of different drugs. Unlike the screening of drugs such as antibiotics, antithrombotic drugs require multi-
parametric endpoint analysis. Animal models are the most useful system in the evaluation of the effects of these drugs.

Finally, it should be stressed that the evaluation of pharmacopoeial and in vitro potency of antithrombotic drugs does not necessarily reflect the in vivo safety/efficacy profile. Endogenous modulation, such as the release of tissue factor pathway inhibitor (TFPI) by heparins, plays a very important role in the overall therapeutic index of many drugs. Such data can only be obtained using animal models. It is therefore important to design experiments where several data points can be obtained. This information is of crucial value in the assessment of antithrombotic drugs and cannot be substituted by other in vitro or tissue culture-based methods.

Several excellent reviews covering the different theoretical and technical aspects of thrombosis and thrombolysis models have been published previously (2, 11–15). These reviews, along with more specialized reviews of models of atherosclerosis (16), restenosis (17), and stroke (18) provide comprehensive information regarding the details of many models and provide the pathological rationale for using specific models for specific diseases. In addition, the advantages and disadvantages of each model of thrombosis and thrombolysis are described in these reviews. This chapter focuses on the use of thrombosis models in the drug discovery process, with emphasis on the practical application of these models. Examples from studies evaluating therapeutic approaches that target various antithrombotic mechanisms will be presented to demonstrate the current use of thrombosis models in drug discovery. Important issues in evaluating novel antithrombotic compounds will also be addressed. In addition, evidence demonstrating the clinical relevance of preclinical data derived from animal models of thrombosis will also be presented. Finally, a summary of the use of genetic models of thrombosis/hemostasis and their current and potential use in drug discovery will also be discussed.

2. Animal Models of Thrombosis

2.1. Stenosis- and Mechanical Injury-Induced Coronary Thrombosis (Folts Model)

2.1.1. Purpose and Rationale

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina,
acute myocardial infarction, or sudden death. Treatment with angioplasty, thrombolysis, or bypass grafts can expose new thrombogenic surfaces, and re-thrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In 1976, Folts and co-workers (19) described a model of repetitive thrombus formation, or cyclic flow reductions (CFRs), in stenosed coronary arteries of open-chest, anesthetized dogs (Fig. 2.1). This model is also applicable to the rabbit femoral or carotid artery (20). Using this model, several groups have described the antithrombotic effects of a variety of drugs, primarily prostaglandin-inhibitors, prostacyclin-mimetics, or fibrinogen receptor antagonists (21–27). The combination of two thrombogenic stimuli leads to the development of CFRs in this model: severe, concentric stenosis and focal, intimal injury. With few exceptions, CFRs will not develop unless both stimuli exist.
The rheological conditions required to produce turbulence and stasis upon vessel narrowing dictate that lumenal diameter be reduced by at least 50%. Two- to three-millimeter long constrictors are cut from Lexan® rods readily available from local plastics distributors. One center hole of varying diameter and two smaller collar holes are drilled, into which the prongs of snap-ring pliers fit to spread the constrictor's slit in the top-central portion to apply or remove them. Other plastics will suffice, but Lexan is ideal because of its strength and resiliency. Both circumflex and left anterior descending (LAD) coronary arteries have been used in this model. Besides personal preferences, we know of no physiologic basis for preferring one to the other.

Owing to the prominent auto-regulation of coronary circulation, it is difficult to assess the severity of a stenosis on the basis of changes in basal coronary blood flow (CBF). However, the robust reactive hyperemia (RH) characteristic of the coronary circulation provides a powerful tool with which to gauge the severity of the stenosis. With gradual narrowing, basal CBF will remain unchanged or decline negligibly, whereas RH will begin to decline sooner as the vasodilatory reserve of downstream vessels is progressively exhausted. Reduction of lumenal diameter to this degree is required if one wishes to produce CFRs in a high percentage (i.e., >90%) of dogs. It is also critical if one wishes to compare two or more drugs in this model and draw meaningful conclusions about drug effects. Inasmuch as the severity of the stenosis is an important component of the thrombogenic stimulus, comparable and uniform degrees of constriction between treatment groups are required, preferably those in which basal CBF is reduced between 10 and 25% and RH is abolished, or nearly so. It is important to apply these criteria when one is investigating a drug that possesses vasodilatory effects or one whose pharmacologic profile is not completely known. Without exhaustion of the vasodilatory reserve (as evidenced by abolition of RH), elimination of CFRs could result (at least partly) from coronary vasodilation. One difficulty in using RH or basal flow reduction immediately after placing a constrictor on the coronary artery is that CBF starts to decline quickly as platelets accumulate at the site of stenosis and intimal injury. Thus, one needs to assess the degree of flow reduction immediately after constricting the artery. Delaying this assessment will result in an overestimation of the stenosis severity due to accumulation of platelets on the vessel lining. Alternatively, the degree of stenosis can be ascertained by applying the constrictor before denuding the artery (see below), in which case the constrictor (or constrictors) needs to be removed and reapplied.

After damaging and stenosing the coronary artery sufficiently, CBF starts declining immediately, reaching zero within 4–12 min, and remaining there until blood flow is restored by manually
shaking loose the thrombus ("SL," see Fig. 2.1, bottom). This is usually accomplished by either flicking the Lexan constrictor or sliding the constrictor up and down the artery to mechanically dislodge the thrombus. Spontaneous flow restorations occur under three circumstances: (1) non-severe conditions (i.e., minimal stenosis or de-endothelialization); (2) waning CFRs (which can occur as late as 30–45 mm after establishing CFRs); and/or (3) administration of a partially effective antithrombotic agent.

Although the influence of blood pressure on the rate of formation of occlusive thrombi or their stability has not been studied systematically, one might predict that higher arterial pressures would increase the deceleration of CBF to zero by enhancing platelet aggregation through increased shear forces and increased delivery of platelets to the growing thrombus. Higher arterial pressure also might increase the propensity for spontaneous flow restorations before an occlusive thrombus is formed, due to greater stress on the nascent, unconsolidated thrombus.

Several groups have examined histologically the coronary arteries harvested from dogs undergoing CFRs usually when CBF is declining or has ceased. Extensive intimal injury, including de-endothelialization with adherent platelets and/or microthrombi, is consistently observed. Arteries harvested when CBF is zero invariably reveal a platelet-rich thrombus filling the stenotic segment (19, 21, 23, 28). These histological observations, coupled with the pattern of gradual, progressive declines in CBF and abrupt increases thereof (whether spontaneous or deliberate), provide further evidence that CFRs indeed are caused primarily by platelet thrombi, not vasoconstriction.

Although the primary cause of CFRs is platelet aggregation, it is possible that local vasospasm and/or vasoconstriction downstream from the site of thrombosis are induced by vasoactive mediators released by activated and/or aggregating platelets. Experimental evidence supporting vasoconstriction just downstream from the stenosis during CFRs has been demonstrated (29).

Further evidence for platelet-dependent thrombus formation in the etiology of CFRs is derived from the pharmacological profile of this model. In general, platelet-inhibitory agents consistently abolish or attenuate CFRs, whereas vasodilators (e.g., nitroglycerin, calcium entry blockers, and papaverine) affect them negligibly (30). Aspirin was the first described inhibitor of CFRs (21). However, in subsequent studies, its effects on CFRs were found to be variable and dose-dependent (22). Variability in the response to aspirin may be related to the severity of the stenosis, as further tightening of the constrictor after an effective dose of aspirin or ibuprofen usually restores CFRs.

Prostacyclin, a powerful anti-aggregatory and potent coronary vasodilatory product of endothelial arachidonic acid
metabolism, is extremely efficacious and potent in abolishing CFRs. It is noteworthy that different drug classes can be compared, as evidenced by the wide range of percentages of responders (28).

Advances in platelet physiology and pharmacology have identified a new class of antiplatelet agents that block the platelet membrane glycoprotein IIb/IIIa (GPIIb/IIa) receptor and hence fibrinogen binding. Fibrinogen binding between platelets is an obligate event in aggregation and is initiated by blood-borne platelet agonists such as ADP, serotonin, thrombin, epinephrine, and collagen (31). The tripeptide sequence Arg–Gly–Asp (RGD), which occurs twice in the Aα-chain of fibrinogen, is believed to mediate, at least in part, the binding of fibrinogen to the GPIIb/IIa complex.

Early experimental results with GPIIb/IIa antagonists in studies by Coller et al. (32), Bush (26), and Shebuski (25, 33) demonstrated that fibrinogen receptor antagonists are as effective as prostacyclin as anti-aggregatory and antithrombotic agents and do not possess the hemodynamic liabilities associated with prostaglandin-based compounds. Monoclonal antibodies directed against the platelet fibrinogen receptor (abciximab) are essentially irreversible, whereas RGD (tirofiban) or KGD (eptifibatide)-based fibrinogen receptor antagonists are reversible, their effects dissipating within hours after discontinuation of intravenous infusion.

The prominence of platelet aggregation vis-à-vis coagulation mechanisms in the Folts model is evidenced by the lack of effect of heparin and thrombin inhibitors reported by most investigators (19, 22). However, heparin and MCI-9038, a thrombin inhibitor, were reported to abolish CFRs in about two-thirds of dogs with recently (30 min) established CFRs, but not in those extent after 3 h (34). The explanation for the differential effects of heparin is not immediately apparent. It may be related to the severity of the stenosis used. These apparently discrepant observations could be related to inhibition of thrombin-stimulated platelet activation and/or aggregation.

An attractive feature of the Folts model is its amenability to dose-response studies. Unlike other models in which the thrombotic processes are dynamic, occurring over several minutes to hours, CFRs in the Folts model are repetitive and remarkably unchanging. In the many dogs that received either no intervention or vehicle 1 h after initiating CFRs, flow patterns remained unchanged for at least another hour (23). Thus, one can evaluate several doses of an investigational drug in a single dog. We and others have exploited this to determine potencies, an important basis of comparison between drugs with similar mechanisms of action, thus underscoring another feature of the model: its amenability to quantification of drug response. Two methods for quantifying drug effects in this model have been described.
Aiken et al. (21) first described a four-point scoring scheme to assess and compare different doses or drugs, ranging from 0 (no effect on CFRs) to 3 (fully effective; complete abolition of CFRs). Intermediate scores of 1 and 2 are respectively applied when the CFR frequency was slowed (but occlusive thrombi still occurred) and when non-occlusive, spontaneously embolizing thrombi were observed. An advantage of this system is the provision of a single number for each evaluation period. A disadvantage is that agents that decrease systemic blood pressure (e.g., prostacyclin) will also decrease coronary perfusion pressure; the coronary flow pattern will be affected, making the scoring system somewhat more subjective.

Another method of quantifying CFRs, described by Bush et al. (23), addresses the frequency, expressed on a per hour basis, and severity, based on the average nadir of CBF before a flow restoration. This system is less subjective, but it produces two values per evaluation period, and combinations of the two in an effort to provide a single parameter are awkward. In practical terms, both methods for quantifying CFRs described above provide similar answers. The important point for both is consistency in scoring. This end is best served by well-defined and communicated criteria.

To date, the Folts model has been used only to evaluate antithrombotic drugs. No description of this model for the evaluation of thrombolytic drugs or adjunctive agents has been made. However, preliminary data reveal these thrombi to be resistant to doses of thrombolytic agents that lyse thrombi in other models (35). Of all the models described in this review, the thrombi in this model are probably the most platelet-rich and possess relatively less fibrin than, for example, the copper coil or wire models. However, it may be erroneous to conclude that these thrombi are devoid of fibrin, as the fibrinogen that links platelets during aggregation via the GPIIb/IIIa receptor is theoretically capable of undergoing fibrin formation.

Several investigators have shown that the same combination of severe vessel narrowing and de-endothelialization results in CFRs in arteries other than the coronary. We have elicited CFRs in femoral arteries in anesthetized dogs with similar degrees of vessel narrowing and deliberate denudation of the artery (unpublished observation). Folts et al. (24) demonstrated that CFRs can be produced in conscious dogs with chronically implanted Lexan® coronary constrictors and flow probes. CFRs were prevented in the interim between implantation and acute study by the administration of aspirin. Al-Wathiqui (36) and Gallagher and co-workers (37) have demonstrated that progressive carotid or coronary arterial narrowing with ameroid constrictors will result in CFRs days to weeks after surgical implantation. These dogs apparently did not undergo deliberate vessel denudation at the
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Perhaps focal inflammation developed in the intervening week(s) between the surgery and the development of CFRs in these animals. Alternatively, there was sufficient intimal vessel injury during implantation of the ameroid constrictors to induce development of CFRs at a later time. CFRs have also been elicited in the renal (38) and carotid (39) arteries of cynomolgus monkeys.

Eidt et al. (40) showed that conscious dogs equipped with the same constrictors over segments of the LAD showing endothelial injury undergo repetitive CFRs in response to exercise, but not ventricular pacing. The frequency and severity of CFRs varied more in this model, and some CFRs were non-occlusive. CFRs of most dogs eventually deteriorated to persistent no- or low-flow states. Unlike the open-chest preparation, flow restorations observed in this model occurred spontaneously. Also, the severity of the stenosis produced was not as great as that produced by most practitioners of the Folts model, as reflected by the ability of CBF to increase above control levels initially during exercise.

In summary, the Folts model of platelet-dependent thrombus formation is a well-established method to determine the pharmacology of antithrombotic agents. It represents an excellent choice for initial evaluation of antiplatelet activity in vivo, regardless of the artery used. Qualitatively, the thrombogenic stimuli in the Folts model and those responsible for unstable angina may be similar, since an involvement by platelets has clearly been demonstrated in the model and is strongly suspected clinically. It should be remembered, however, that flow restorations in the Folts model require vigorous shaking. In contrast, unstable angina is believed not to involve persistent, total thrombotic coronary occlusion. On the basis of the model’s pharmacological profile, the thrombi in this model also do not appear to resemble those usually responsible for acute myocardial infarction, as the former appear to be unresponsive to thrombolytic agents. The preliminary observations that either streptokinase (SK) or tissue plasminogen activator (t-PA) does not lyse thrombi in the Folts model contrast with the 50–75% response rate to thrombolytic therapy in patients with evolving myocardial infarction (35). However, it is tempting to speculate that the platelet-rich thrombi produced in this model are more like thrombi in those patients whose coronary arteries are not reopened by even early intervention and/or high doses of t-PA (41), and thus could represent a model of “thrombolytic-resistant” coronary thrombosis.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts and Rowe (42) developed a model of periodic acute platelet thrombosis and CFRs in stenosed canine coronary arteries. Uchida described a similar model in 1975 (27). The model includes various aspects of unstable angina pectoris, i.e., critical stenosis, vascular damage, downstream vasospasm.
induced by vasoconstrictors released or generated by platelets. The cyclic variations in CBF are a result of acute platelet thrombi which may occlude the vessel but which either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. CFRs are not a result of vasospasm (24). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases but its effect is limited. Similarly, CFRs in the Folts model are abolished by aspirin but the effect can be reversed by increases in catecholamines and shear forces (43). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (44).

In this section, five different protocols are described for the induction of coronary thrombosis.

The first four protocols are characterized by episodic, spontaneous decreases in CBF interrupted by restorations of blood flow. These alterations in CBF, or CFRs, are associated with transient platelet aggregation at the site of the coronary constriction and an abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage to the vessel wall is achieved by placing a hemostatic clamp on the coronary artery. A fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder at the damaged part of the vessel. In dogs, stenosis is critical; the reactive hyperemic response to a 10-second (s) occlusion is abolished (protocol 1). In pigs, stenosis is subcritical; partial reactive hyperemia remains (45).

For some animals, particularly young dogs, damage of the vessel wall and stenosis are not sufficient to induce thrombotic cyclic flow variations. In these cases, additional activation of platelets by infusion of epinephrine (protocol 3) is required, leading to the formation of measurable thrombi. In protocol 4, thrombus formation is induced by subcritical stenosis without prior clamping of the artery and infusion of platelet activating factor (PAF), according to the model described by Apprill et al. (46). In addition to these protocols, coronary spasms induced by released platelet components can influence CBF. Therefore, this model includes the main pathological factors of unstable angina pectoris.

In this protocol, coronary thrombosis is induced by delivery of low amperage electrical current to the intimal surface of the artery, as described by Romson et al. (47). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours. As a consequence of the time course, thrombi formed are of mixed type and contain more fibrin than platelet thrombi formed by critical stenosis.
2.1.2. Procedure

2.1.2.1. Protocol 1: Critical Stenosis

Dogs of either sex weighing 15–40 kg and at least 8 months of age are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and then continuous infusion of approximately 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space; the pericard is opened and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flow probe is placed on the proximal part of the LCX to measure CBF. Distal to the flow probe, the vessel is squeezed with a 2-mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor 2–4 mm in length and with an internal diameter of 1.2–1.8 mm (depending on the size of the LCX) is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (2–5 times) until the appropriate narrowing of the vessel is achieved and cyclic flow variations are observed. In the event of occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site.

Only dogs with regularly spaced CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments. The test substance is administered by i.v. bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2–4 × 60 min and compared to pre-treatment values. Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

2.1.2.2. Protocol 2: Subcritical Stenosis

Male castrated pigs (German landrace weighing 20–40 kg) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.), and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space; the pericard is opened and the LAD is exposed. An electromagnetic or Doppler flow probe is placed on the proximal part of the LAD to measure CBF. Distal to the flow probe, the vessel is squeezed with a 1-mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor 2 mm in length is then placed around the artery at the site of damage. Usually, the constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved that produces CFRs. CFRs are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization
does not occur spontaneously, the formed thrombus is released by reducing blood flow by shortly lifting the vessel with forceps.

Only pigs with regularly spaced CFRs of similar intensity within a pre-treatment phase of 60 min are used for the experiments. The test substance is administered by i.v. bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for $2 \times 60$ min and compared to pre-treatment values.

2.1.2.3. Protocol 3: Stenosis Plus Epinephrine Infusion

If protocol 1 does not lead to CFRs, additional epinephrine (0.2 $\mu$g/kg/min) is infused into a peripheral vein for $2 \times 60$ min (60 min before and 60 min after drug administration). CFRs are recorded, and the 60 min post-drug phase is compared to the 60 min pre-drug phase.

2.1.2.4. Protocol 4: Stenosis Plus PAF Infusion

The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus formation (subcritical stenosis). For the induction of CFRs, PAF (C16-PAF, Bachem) (0.2 nmol/kg/min) is infused into one cannulated lateral branch of the coronary artery. After 30 min, PAF infusion is terminated and blood flow returns to a normal, continuous course. Thirty minutes later, the test substance is administered concomitantly with the initiation of a second PAF infusion for 30 min. CFRs are recorded and the drug treatment/second PAF phase is compared to the pre-drug/first PAF phase.

2.1.2.5. Protocol 5: Electrical Stimulation

The LCX is punctured distal to the flow probe with a chrome-vanadium-steel electrode (3 mm in length, 1 mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a teflon-coated wire to a 9-volt (V) battery, a potentiometer, and an amperemeter. A disc electrode (cathode) is secured to a subcutaneous thoracic muscle layer to complete the electrical circuit. The intima is stimulated with 150 $\mu$A for 6 hours (h). During this time, an occluding thrombosis is gradually formed.

The test substance, or vehicle as a control, is administered either at the start of the electrical stimulation or 30 min after the start. The time until thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) are determined. Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

For all protocols the following preparations and measurements are performed:

1. To measure peripheral arterial blood pressure (BP) [mm Hg], the right femoral artery is cannulated and connected to a Statham pressure transducer.
2. Left ventricular pressure (LVP) [mm Hg] is determined by inserting a micro tip catheter via the carotid artery retrogradely.

3. Left ventricular end-diastolic pressure (LVEDP) [mm Hg] is evaluated through sensitive amplification of the LVP.

4. Contractility (LV $\frac{dp}{dt}$ max) [mm Hg/s] is determined from the initial slope of the LVP curve.

5. Heart rate [min$^{-1}$] is determined from the pulsatile blood pressure curve.

6. The ECG is recorded in lead II.

7. Arterial pH and concentrations of blood gases are maintained at physiological levels by adjusting respiration and infusion of sodium bicarbonate.

8. Blood hematocrit values (37–40%) and number of erythrocytes are kept constant by infusion of oxypolygelatine in dogs and electrolyte solution in pigs.

9. Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic temperature regulation.

10. Template buccal mucosal bleeding time is carried out using the Simplate device.

11. At the end of the test, animals are sacrificed by an overdose of pentobarbital sodium.

For detailed applications of the Folts model, see Folts (44), Folts and Rowe (42, 43), and Folts et al. (19, 24).

2.1.3. Evaluation

For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic) [mm Hg] is determined.

2.1.3.1. Protocols 1–4

The following parameters are determined to quantify stenosis-induced coronary thrombosis:

1. Frequency of CFRs = cycle number per unit time

2. Magnitude of CFRs = cycle area [$mm^2$] (total area of all CFRs per unit time, measured by planimetry)

3. Percent change in cycle number and cycle area after drug treatment is calculated relative to pre-treatment controls.

4. Statistical significance is assessed by the paired Student’s $t$-test.

2.1.3.2. Protocol 5

The following parameters are determined to quantify electrically induced coronary thrombosis:

1. Occlusion time [min] = time to zero blood flow.

2. Thrombus size [mg] = wet weight of the thrombus immediately after removal.
3. Percent change in mean values for occlusion time and thrombus size in drug-treated groups is calculated relative to the control group.

4. Statistical significance is assessed by the non-paired Student’s $t$-test.

2.1.4. Critical Assessment of the Method

The stenosis (Folts) and electrical (Romson/Lucchesi) models of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush and Patrick (28) provide an excellent review of the role of the endothelium in arterial thrombosis and the use of the Folts model to determine the effects of thrombosis inhibitors and mediators, i.e., thromboxane, prostacyclin, cyclooxygenase, serotonin, nitric oxide (NO) donors, and other vasodilators. The effect of an NO donor could be reversed by the NO scavenger oxyhemoglobin, which indicated that NO indeed mediates antithrombotic drug action (48). These coronary thrombosis models have recently been used to elucidate the mechanisms of action of several antithrombotic drugs, including the oral GPIIb/IIIa antagonist DMP 728 (49); the low molecular weight heparin (LMWH) enoxaparin (50), which, in contrast to unfractionated heparin, inhibited CFRs; the thrombin inhibitor PEG–hirudin (51); melagatran (52), an anti-P-selectin antibody (53); and activated protein C (54).

The clinical relevance of the Folts model has been questioned because the model is very sensitive to antithrombotic compounds. However, in this model, lack of a reversal by epinephrine or an increase in degree of stenosis is able to differentiate any new drug from aspirin. Electrical coronary thrombosis is less sensitive (i.e., aspirin has no effect) and higher doses of some drugs are required. However, in principle, most drugs act in both models, if at all.

2.1.5. Modifications of the Method

Romson et al. (55) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low amperage electric current to the intimal surface of the artery. Benedict et al. (56) modified the electrical stimulation of thrombosis model by using two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped when a 50% increase in flow velocity was reached, at which point thrombosis occurred spontaneously. Using this model, the investigators demonstrated the importance of serotonin by measuring increased coronary sinus serotonin levels just prior to occlusion.

Warltier et al. (57) described a canine model of thrombin-induced coronary artery thrombosis to analyze the effects of intracoronary SK on regional myocardial blood flow, contractile function, and infarct size. Al-Wathiqi et al. (36) described
the induction of CFRs in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs. The Folts thrombosis model has also been applied to carotid arteries in monkeys. Coller et al. (58) induced CFRs in the carotid arteries of anesthetized cynomologus monkeys and demonstrated that they were abolished by the GPIIb/IIIa antibody abciximab.

2.2. Stenosis- and Mechanical Injury-Induced Arterial and Venous Thromboses (Harbauer Model)

2.2.1. Purpose and Rationale

Harbauer (59) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification of the technique, both arterial and venous thromboses are produced in rabbits by stenosis of the carotid artery and the jugular vein with simultaneous mechanical damage of the endothelium. This results in the activation of platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed and detected by blood flow measurements. The dominant role of platelets in this model is evidenced by the inhibitory effect of an antiplatelet serum in both types of vessels (60). The modified Harbauer model is used to evaluate the antithrombotic activity of compounds in an in vivo model of arterial and venous thromboses in which thrombus formation is highly dependent on platelet activation.

2.2.2. Procedure

Male Chinchilla rabbits weighing 3–4 kg receive test compound or vehicle as a control by oral, i.v., or i.p. administration. The first ligature (vein; for preparation, see below) is performed at the end of the absorption period (i.p., approximately 30 min; p.o., approximately 60 min; i.v., variable).

Sixty-five minutes before stenosis, the animals are sedated by i.m. injection of 8 mg/kg xylazine (Rompun®) and anesthetized by i.v. injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein.

A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube. One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow around the vessel occlusion.
Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as centered as possible). Blood flow [ml/min] is measured continuously.

After blood flow reaches a steady state (approximately 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching) and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approximately 1.5 min), the carotid artery is damaged by briefly squeezing it with forceps. A small plastic constricting cylinder (2 mm in length and 1.2 mm in diameter) is placed around the site of endothelial damage.

Template bleeding time is measured at various time intervals before and after drug treatment (depending on the route of administration) in the shaved inner ear using a Simplate® device. Care is taken to select parts of the skin without large vessels.

2.2.3. Evaluation

1. Percent thrombus formation (thrombosis incidence) is determined as the number of occluded vessels (blood flow=0).
2. Percent inhibition of thrombosis is calculated in each dosage group relative to the respective vehicle controls.
3. Thrombosis incidence in the vehicle controls is set as 100%.
4. Statistical significance is assessed by means of the Fisher-exact-test.
5. If initial values for blood flow do not significantly differ in the dosage and control groups, the area below the blood flow curve is measured by planimetry, and the mean value of each dosage group is compared to the control using the unpaired Student’s t-test.
6. Mean occlusion time [min] in the dosage and control groups are calculated and compared using the Student’s t-test.
7. The maximal change in systolic and diastolic blood pressure during the time period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. For example, a reduction of systolic blood pressure by 30 mmHg and diastolic blood pressure by 20 mmHg is generally accepted as a strong reduction in blood pressure.

2.2.4. Critical Assessment of the Method

Two main factors of arterial thrombosis in human are essential components of this model: high-grade stenosis and vessel wall damage. In the absence of either, no thrombus is formed. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels, thrombus formation is dependent on platelet function, as shown by the effects of antiplatelet serum.
Thus, jugular vein thrombosis in this model differs from stasis-induced deep vein thrombosis with prominent fibrin formation. On the other hand, occlusive thrombi are more stable than the pure platelet thrombi in the Folts model (see Section 2.1), as carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs have been shown to be effective in this model: (1) antiplatelet drugs such as ticlopidine, prostacyclin/iloprost, NO donors (SNP, molsidomine), but not aspirin or thromboxane synthetase inhibitors; (2) anticoagulants such as hirudin, high-dose heparin, and warfarin; and (3) SK/t-PA (60, 61, and unpublished data). Drugs that simply lower blood pressure, such as hydralazine, clonidine, and prazosin have no effect on thrombus formation in this model.

Bevilacqua et al. (61) applied this model to the rabbit carotid arteries and compared one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH$_2$Cl, iloprost, and t-PA, but not aspirin, inhibited carotid occlusion in this model.

Spokas and Wun (62) induced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. Vascular wall damage was achieved by crushing the vessel with hemostat clamps. A segment of the vena cava was looped with two ligatures 2.5-cm apart, and then 2 h after ligation, the isolated venous sac was dissected and the clot was removed for determination of dry weight.

Lyle et al. (63), in pursuit of an animal model that mimicked thrombotic re-occlusion and restenosis after successful coronary angioplasty in human, developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute $^{111}$indium-labeled platelet deposition and thrombosis were assessed 4 h after balloon injury in arteries subjected to prior endothelial damage (by air desiccation) and cholesterol supplementation (one month). The effects of inhibitors of FXa or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

Meng (64), Meng and Seuter (65), and Seuter et al. (66) described a method to induce arterial thrombosis in rats by chilling of the carotid artery (thrombosis induced by super cooling). Rats were anesthetized, and then the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove that was cooled to $-15^\circ$C. The vessel was then compressed using a weight of 200 g. In addition, a silver clip was fixed to the vessel distal to the injured area to produce disturbed and slow blood flow. After 4 min, the proximal clamp was removed and blood flow was reestablished in the injured artery. A similar model in the rabbit has also been developed, with slightly different conditions (chilling temperature of $-12^\circ$C for 5 min, and a compression weight of 500 g). The wound is closed and the animal is allowed to recover from
anesthesia. Antithrombotic compounds are administered in various doses at different time intervals before surgery. After 4 h, the animals receive heparin and are re-anesthetized. The lesioned carotid artery is removed and thrombus wet weight is immediately measured.

2.3. Electrically Induced Thrombosis

A novel technique for inducing arterial thrombosis was introduced by Salazar (67) in which anodal current was delivered to the intravascular lumen of a coronary artery in the dog via a stainless steel electrode. The electrode was positioned under fluoroscopic control, which somewhat complicated the procedure. Subsequently, Romson et al. (55) modified the procedure such that the electrode was placed directly into the coronary artery of an open-chest, anesthetized dog. This technique then allows one to produce a thrombus in the anesthetized animal or to close the chest after inserting the electrode and allow the animal to recover, after which thrombosis can be elicited later in the conscious animal. The advantage of this modification is that it allows induction of thrombus formation without the need for fluoroscopy.

The stimulation electrode is constructed from a 25- or 26-gauge stainless steel hypodermic needle tip, which is attached to a 30-gauge teflon-insulated silver-coated copper wire. Anodal current is delivered to the electrode via either a 9-V nickel–cadmium battery with the anode connected in series to a 250,000-ohm potentiometer or with a Grass stimulator connected to a Grass constant current unit and a stimulus isolation unit. The cathode in both cases is placed into a subcutaneous site completing the circuit. The anodal current can be adjusted to deliver 50–200 μA. Anodal stimulation results in focal endothelial disruption, which in turn induces platelet adhesion and aggregation at the damaged site. This process is then followed by further platelet aggregation and consolidation, with the growing thrombus entrapping red blood cells.

A modification of the method of Romson et al. (55) involves placement onto the coronary artery of an external, adjustable occluder (68) to produce a fixed stenosis on the coronary artery. A flow probe to record CBF is placed on the proximal portion of the artery followed by the stimulation electrode, with the clamp being placed most distally (Fig. 2.2). The degree of stenosis can then be controlled by adjusting the clamp. The resulting stenosis is produced in an effort to mimic the human pathophysiology of atherosclerotic coronary artery disease, whereby thrombolytic therapy restores CBF through a coronary artery with residual narrowing due to atherosclerotic plaque formation.

Another modification of the electrical stimulation model that merits discussion is described by Benedict et al. (56). They discontinued anodal current when mean distal coronary flow velocity (measured with Doppler flow meter) increased by approximately
50%, reflecting disruption of normal axial flow by the growing thrombus. Occlusive thrombosis occurred within 1 h after stopping the current (2 h after starting the current). In these studies, coronary sinus plasma levels of serotonin, an index of intravascular platelet aggregation, were increased approximately 20-fold just before occlusive thrombus formation. The results of these studies agree with others in showing that either proximal flow velocity or electromagnetically measured CBF declines trivially over the majority of the time period in which the thrombus is growing. The largest declines in (volume) flow occur over a small and terminal fraction of the period between initial vessel perturbation and final occlusion. During that interval, coronary lumenal area decreases rapidly and to a critical degree, as platelets accrue at the growing thrombus. The studies by Benedict et al. (56) demonstrate that this final phase of thrombosis can occur independently of electrical stimulation. This variation of the model may be attractive to those who wish to produce occlusive thrombosis without continued electrical stimulation.
Regardless of whether electrical stimulation is continued until occlusive thrombosis, there is another component to this model that has upside and downside potential, namely, the opportunity for coronary vasoconstriction to occur. Although the incidence of Prinzmetal’s angina is low, it is widely suspected that vasospasm superimposes on a primarily thrombotic event in unstable angina and myocardial infarction. In studies by Van der Giessen et al. (69), nifedipine was reported to increase the extent of CBF after plasmin-induced thrombolysis in a porcine model of electrically induced coronary thrombosis. In their model, the anodal stimulation was applied circumferentially to the exterior surface of the LAD, and an external constrictor was not used.

Depending on the hypothesis being tested, the experimenter can leave intact or minimize this potential through the use or disuse of an external constrictor. As in the Folts and Gold coronary thrombolyis models, blood pressure must be taken into account or maintained within acceptable limits, since, in the presence of a critical stenosis, auto-regulation no longer exists. Under these conditions CBF is highly dependent on driving pressure (arterial pressure).

Numerous experimental studies evaluating anticoagulants, antithrombotic, and/or thrombolytic drugs have been performed using this model. In the initial report by Romson et al. (55), the cyclooxygenase inhibitor ibuprofen was evaluated. Comparison of myocardial infarct size, thrombus weight, arrhythmia development, and scanning electron microscopy of drug-treated and control animals indicated that ibuprofen protected the conscious dog against the deleterious effects of coronary artery thrombosis. Subsequent studies in the same model and laboratory evaluated the antithrombotic potential of various TXA2 synthetase inhibitors, such as U 63557A, CGS 13080, OKY 1581, and dazoxiben. When the TXA2 synthetase inhibitors were administered before induction of the current, OKY 1581 (70) and CGS 13080 (71) reduced the incidence of coronary thrombosis, whereas U 63557A (72) and dazoxiben (73) were ineffective and partially effective, respectively. The differences in efficacy noted among the TXA2 synthetase inhibitors were ascribed to differences in potency and duration of action.

Other investigators have used this model to study the prevention of original coronary thrombosis in the dog. Fitzgerald et al. (74) studied the TXA2 synthetase inhibitor U 63557A alone or in combination with L-636,499, an endoperoxide/thromboxane receptor antagonist. U 63557A alone did not prevent coronary thrombosis when administered before current application, whereas the combination of U 63557A and L-636,499 was highly effective. These data suggest that prostaglandin endoperoxides may modulate the effects of TXA2 synthetase inhibitors and that this response may be blocked by concurrent administration of
an endoperoxide/thromboxane receptor antagonist. The murine monoclonal antibody to platelet GPIIb/IIIa (7E3) was studied in this model for its ability to prevent thrombus formation. At a dose of 0.8 mg/kg i.v., the 7E3 monoclonal antibody completely prevented original thrombus formation (75).

In addition to the evaluation of antithrombotic (i.e., antiplatelet) agents, the electrical injury model is useful for studying anticoagulant FXa inhibitors, such as YM 60628 (86), and thrombolytic drugs. When evaluating thrombolytic agents, the thrombus is allowed to form without drug intervention and then aged for various periods. Schumacher et al. (68) demonstrated that intracoronary SK was an effective thrombolytic drug in this model; the thrombolytic effectiveness being augmented by the concurrent administration of heparin and prostacyclin, or by a TXA2 synthetase inhibitor (68). In other studies reported by Shebuski et al. (76), the TXA2 receptor antagonist BM 13.177 hastened t-PA-induced thrombolysis and prevented acute thrombotic re-occlusion. Van der Giessen et al. (77) subsequently demonstrated that BM 13.177 prevented original thrombus formation in 75% of pigs undergoing electrical stimulation; aspirin was ineffective in this porcine model. These and other studies underscore the potential for adjunctive therapy to hasten thrombolysis and/or prevent re-occlusion, both contributing to greater salvage of ischemic myocardium.

Like the copper coil model, the electrical stimulation model has been used to produce experimental myocardial infarction. Patterson et al. (78) have used this technique to produce coronary thrombosis in the LCX (which supplies blood flow to the posterior LV wall in dogs) in dogs with a previous anterior wall infarct to mimic sudden cardiac death that occurs in people during a second (recurrent) myocardial infarction or ischemic event.

This model has also been modified to demonstrate the efficacy of adjuncts to thrombolytic therapy (79–82). In this case, the thrombus is allowed to extend until it completely occludes the vessel. Usually, the thrombus is allowed to stabilize, or “age,” to mimic the clinical setting in which a time lag exists between the thrombotic event and the pharmacological intervention. At the end of the stabilization period, thrombolytic agents such as t-PA or SK are administered in conjunction with the novel antithrombotic agent to lyse the thrombus and maintain vessel patency. The incidence and times of reperfusion and re-occlusion are the major endpoints. These studies have established that recombinant tick anticoagulant peptide (rTAP), a potent and selective FXa inhibitor derived from the soft tick (83), promotes rapid and prolonged reperfusion at doses that produce relatively minor elevations in PT, aPTT, and template bleeding time.
2.3.1. Purpose and Rationale

The use of an electrical current to induce thrombosis in hamster and dog was described in the early 1950s by Lutz et al. (84) and Sawyer et al. (85, 86). In general, two different approaches are taken in this model. One method produces electrical damage by means of two externally applied hook-like electrodes (87, 88). The other method uses a needle electrode that is advanced through the walls of the blood vessel and positioned in the lumen; a second electrode is placed at a subcutaneous site to complete the circuit (55, 56, 67).

2.3.2. Procedure

Anaesthetized rats weighing 200–300 g are intubated and a femoral artery is cannulated for administration of test compound(s). One carotid artery is isolated from the surrounding tissue over a distance of 10–15 mm.

A pair of rigid stainless steel wire hook-like electrodes with a working distance of 4 mm is positioned on the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flow meter (Transonic, Ithaca NY, USA); the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimulator (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery.

2.3.3. Evaluation

1. Blood flow before and after induction of thrombus for 60 min.
2. Time to occlusion [min] = the time between onset of the electrical current and the time at which blood flow decreases to less than 0.3 ml/min.
3. Patency of the blood vessel over 30 min.

2.3.4. Critical Assessment of the Method

Thrombi formed by electrical induction are composed of densely packed platelets, with some red cells. Moreover, electrical injury causes extensive damage to intimal and sub-intimal layers. The endothelium is completely destroyed, and the damage extends to sub-endothelial structures, including smooth muscle cells. This deep damage could reduce sensitivity in terms of discriminating between drugs on the basis of their antithrombotic activity. However, Philp et al. (88) showed that unfractionated heparin completely blocks thrombus formation, whereas other antiplatelet agents exhibit differential antithrombotic actions. The investigators concluded that this relatively simple model of arterial thrombosis might prove to be a useful screening test for drugs with antithrombotic potential.
2.3.5. Modifications of the Method

In a modification of this model by Salazar (67), a stainless steel electrode is inserted into a coronary artery in the dog to deliver anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control, complicating the procedure somewhat. Romson et al. (55) described a further modification in which the electrode was placed directly into the coronary artery of open-chest anaesthetized dogs.

Rote et al. (89, 90) applied the carotid thrombosis model to dogs. A calibrated electromagnetic flow meter was placed on each common carotid artery proximal to the point of insertion of an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern was decreased by 25% without alteration in mean blood flow. Electrolytic injury to the intimal surface was accomplished with an intravascular electrode composed of a teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V nickel–cadmium battery in series with a 250,000-ohm variable resistor. The cathode was connected to a subcutaneous site. Injury was initiated in the right carotid artery by application of a 150-μA continuous pulse anodal direct current to the intimal surface of the vessel for a maximum duration of 3 h, or for 30 min beyond the time of complete vessel occlusion, as determined by blood flow recordings. Upon completion of the study on the right carotid, the procedure was repeated on the left carotid artery after administration of test drug.

Benedict et al. (56) introduced a procedure in which anodal current was discontinued when mean distal coronary flow velocity increased by approximately 50%, reflecting disruption of normal flow by the growing thrombus. An occlusive thrombosis occurred within 1 h after cessation of the electrical current. In this model, the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (91). A 10-min anodal electrical current of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle treated ferrets within 41 ± 3 min with an average weight of 8 ± 1 mg. Thrombus weight was reduced by aspirin or a thromboxane receptor antagonist.

Guarini (92) reported the formation of a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

2.4. Ferric Chloride (FeCl₃)-Induced Thrombosis

The administration of a variety of chemicals either systemically or locally can result in damage to the endothelium with
subsequent generation of a thrombus. Such compounds include ferric/ferrous chloride, fluorescein-labeled dextran and Rose Bengal.

In models employing ferric (ferrous) chloride (93), the carotid artery of rats is isolated. A flow probe is placed proximal to the intended site of lesion and a 3-mm disc of filter paper which has been soaked in ferric/ferrous chloride (35-50%) is placed on the artery. The application of ferric (ferrous) chloride results in transmural vascular injury leading to the formation of occlusive thrombi. This injury is believed to be a result of lipid peroxidation catalyzed by the ferric (ferrous) chloride. Thrombus formation, measured as a decrease in blood flow through the vessel, typically occurs within 30 min. Microscopic analysis of the thrombi has shown them to be predominantly platelet-rich clots. This model has been used to study the antithrombotic effects of direct thrombin inhibitors (94–96) and heparins.

Endothelial damage can also be induced by fluorescein- or fluorescein isothiocyanate (FITC)-conjugated compounds. A model has been described in which FITC–dextran is administered intravenously to mice. Thrombus formation is induced upon exposure of the microvessels of the ear to the light of a mercury lamp (excitation wavelength of 450–490 nm) (97). The endothelial damage induced in this model is believed to be a result of the generation of singlet molecular oxygen produced by energy transfer from the excited dye (98). Thrombus formation is measured using intravital fluorescence microscopy. This detection technique allows for a number of endpoints to be quantitated, including changes in luminal diameter due to thrombus formation, blood flow measurements, and extravasation of the FITC–dextran. This model offers the advantages of not requiring surgical manipulations, which can cause hemodynamic or inflammatory changes, allowing for repeated analysis of the same vessel segments over time, and being applicable to the study of both arteriolar and venular thromboses. The administration of Rose Bengal has been used similarly (99).

### 2.4.1. Purpose and Rationale

A variety of chemical agents have been used to induce thrombosis in animals. The use of topical FeCl₃ as a thrombogenic stimulus in veins was described by Reimann-Hunziger (100). Kurz et al. (93) demonstrated that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This simple and reproducible test has been used for the evaluation of antithrombotic (101) and pro-fibrinolytic test compounds (102).

### 2.4.2. Procedure

Rats weighing 250–300 g are anaesthetized with Inactin (100 mg/kg) and a polyethylene catheter (PE-205) is inserted
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into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood sampling and measurement of arterial blood pressure and in the jugular vein for administration of test compounds. The right carotid artery is isolated and an ultrasonic Doppler flowprobe (probe 1RB, Transonic, Ithaca NY, USA) is placed on the vessel to measure blood flow. A small piece of parafilm “M” (American Can Co., Greenwich, CT, USA) is placed under the vessel to isolate it from surrounding tissue throughout the experiment.

The test compound is administered by gavage or as an i.v. injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of a piece of filter paper (2 mm × 5 mm) saturated with 25% FeCl$_3$ to the carotid artery. The paper is allowed to remain on the vessel for 10 min and is then removed. Parameters (see below) are monitored for 60 min after the induction of thrombosis, after which the thrombus is removed and weighed.

2.4.3. Evaluation

1. Blood flow before and after induction of thrombus for 60 min
2. Time to occlusion [min]: the time between FeCl$_3$ application and the point at which blood flow decreases to less than 0.3 ml/min
3. Thrombus weight after blotting the thrombus on filter paper

Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride and fresh blood via a side branch (103).

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with the jaws of forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow (Fig. 2.3). The superficial epigastric artery is cannulated for induction of the thrombus and subsequent infusion of thrombolytic agents. Localized thrombi distal to the lateral circumflex artery with snares approximately 1-cm apart are induced by the sequential injection of thrombin, CaCl$_2$ (1.25 mmol), and a volume of blood sufficient to distend the artery. After 30 min, the snares are released and femoral artery blood flow is monitored for 30 min to confirm total obstruction of flow by the thrombus.

These models are not appropriate for evaluating drugs for their ability to inhibit original thrombosis. However, the model is particularly appropriate for evaluating thrombolytic agents and adjunctive therapies for their ability to hasten and/or enhance lysis or prevent acute re-occlusion after discontinuing administration of a thrombolytic agent.
A canine model of thrombin-induced clot formation was developed by Gold et al. (104) in which localized coronary thrombosis was produced in the LAD. This is a variation of the technique described by Collen et al. (105) who used radioactive fibrinogen to monitor the occurrence and extent of thrombolysis of rabbit jugular vein clots. The vessel was intentionally de-endothelialized by external compression with blunt forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) was also injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 min later and total occlusion was confirmed by selective coronary angiography. This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 h, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.
2.5.2. Procedure

In the initial study described by Gold et al. (104), recombinant t-PA was characterized for its ability to lyse 2-h-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25 μg/kg/min i.v. and resulted in reperfusion times of 40, 31, and 13 min, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis. It is also possible to study the effects of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and a2-antiplasmin, as well as to assess myocardial infarct size. For example, Kopia et al. (106) demonstrated that SK elicited dose-dependent thrombolysis in this model.

Subsequently, Gold et al. (107, 108) modified the model to study not only reperfusion, but also acute re-occlusion. Clinically, re-occlusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45% of patients (109). Thus, an animal model of coronary reperfusion and re-occlusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute re-occlusion.

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA occurs with subsequent re-occlusion. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al. (110) and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute re-occlusion (111). These actions in vivo were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

2.6. Laser-Induced Thrombosis

The physiologic responses to injury in the arterial and venous systems vary in part due to differences in blood flow conditions, leading to different clot compositions. This model of arterial thrombosis is based on the development of a platelet-rich thrombus following laser-mediated thermal injury to the vascular wall. This model was first described by Weichert and Breddin (112). In this model, an intestinal loop of an anesthetized rat is exposed through a hypogastric incision and spread on a microscope stage while being continuously irrigated with sterile physiologic saline. Vascular lesions are induced on small mesenteric arterioles with an argon laser beam (50 mW at microscope, 150-ms duration) directed through the optical path of the microscope. Exposure of the laser beam is controlled by means of a camera shutter. Laser shots are made every minute. Antithrombotic potency is evaluated
in real time by microscopic evaluation of vascular occlusion. The number of laser injuries required to induce a thrombus with a length of at least 1.5 times the inner diameter of the vessel is taken as an endpoint.

The antithrombotic activity of several thrombin inhibitors has been compared to unfractionated heparin using the laser-induced thrombosis model. Each inhibitor was administered intravenously via one of the tail veins and allowed to circulate for 5 min prior to the initiation of the laser-induced lesions. Saline-treated control rats required an average of three laser shots to reach an endpoint. Each thrombin inhibitor produced a dose-dependent antithrombotic effect in this model. In comparing the dose of each agent required to extend the endpoint to six laser shots, heparin was observed to be the most potent antithrombotic agent (0.08 μmol/kg), followed by Ac-(D)-Phe-Pro-boroArg-OH (0.154 μmol/kg) and then hirudin (0.28 μmol/kg). Consistent with the results obtained with these agents in the rabbit jugular vein stasis thrombosis model, D-Me-Phe-Pro-Arg-H exhibited the weakest effects in the laser-induced thrombosis model (2 μmol/kg).

2.6.1. Purpose and Rationale

In this model, thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser-mediated thermal injury to the vascular wall. The procedure can be performed in normal or pretreated (i.e., induced arteriosclerosis or adjuvant arthritis) animals. In this model, thrombus formation is mediated by a dual mechanism of platelet adhesion to the injured endothelial vessel wall and ADP-induced platelet aggregation. Most likely, ADP is released by erythrocytes that are lysed by the laser, based on the observation that erythrocyte hemoglobin strongly absorbs the frequencies of light emitted by the laser beam. A secondary aggregation stimulus following the release of ADP is mediated by the platelets themselves.

2.6.2. Procedure

2.6.2.1. Equipment

1. 4 W argon laser (Spectra Physics, Darmstadt, FRG) with a wave length of 514.5 nm; energy below the objective of 15 mW; duration of exposure, 1/30 or 1/15 s.
2. Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
3. Video camera (Sony, Trinicon tube)
4. Recorder (Sony, U-matic 3/4″
5. Videoanalyzer to determine blood flow velocity
Male Sprague-Dawley, spontaneously hypertensive stroke-prone Wistar or Lewis rats with adjuvant-induced arthritis weighing 150–300 g are used. Alternatively, New Zealand rabbits with atherosclerosis induced by cholesterol feeding for 3 months are used. Animals receive test compound by oral, i.v., i.p. or s.c. administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anaesthetized by i.p. administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60, or 90 min post-dosing. The procedure is carried out in arterioles or venules 13 ± 1 μm in diameter of the fat-free ileocaecal portion of the mesentery. During the procedure, the mesenterium is superfused with a physiological saline solution or degassed paraffin liquid (37°C). The ray of the argon laser is guided into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injury is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 s. The number of injuries necessary to induce a defined thrombus is recorded. All thrombi formed during the observation period with a minimum length of 13 μm or an area of at least 25 μm² are evaluated. The procedure is photographed using a video system.

2.6.2.3. Standard Compounds

- acetylsalicylic acid (10 mg/kg, per os)
- pentoxifylline (10 mg/kg, per os)

For a detailed description and evaluation of various agents and mechanisms, please refer to the following references: Arfors et al. (113); Herrmann (114); Seiffge and Kremer (115, 116); Seiffge and Weithmann (117); and Weichert (118).

2.6.3. Evaluation

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated. Results are typically presented in graph form.

2.7. Photochemical Induced Thrombosis

2.7.1. Purpose and Rationale

In 1977, Rosenblum and Sabban (119) reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein, and demonstrated that in contrast to heparin, aspirin and indomethacin prolonged the time to first platelet aggregation. A detailed study by Herrmann (114) demonstrated that scavengers of singlet oxygen, but not hydroxyl radicals, inhibited platelet
aggregation induced by photochemical reaction. The investigators postulated that excitation of intravascular fluorescein results in the production of singlet oxygen, which damages endothelial cells and leads to platelet adhesion and aggregation.

2.7.2. Procedure

Studies are performed in mesenteric arteries 15–30 μm in diameter in anesthetized rats. After i.v. injection of 0.3 ml of fluorescein isothiocyanate–dextran 70 (FITC–dextran; 10%) (Sigma), arterioles are exposed to ultraviolet light (excitation, 490 nm; emission, 510 nm).

2.7.3. Evaluation

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

2.7.4. Critical Assessment of the Method

In contrast to other thrombosis induction methods, photochemically induced thrombosis is amenable to use in small animals. Thrombi are composed primarily of platelets, however, the primary target of the photochemical insult is endothelial cells through induced oxygen radical damage.

2.7.5. Modifications of the Method

Matsuno et al. (120) reported a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (Rose Bengal, 10 mg/kg i.v.) followed by transillumination with a filtered xenon lamp (wave length, 540 nm). Blood flow was monitored by a pulsed Doppler flow meter. Occlusion was achieved after approximately 5–6 min. Pretreatment with heparin prolonged the time required to interrupt the blood flow in a dose-dependent manner. This model has also been used to study the thrombolytic mechanisms of t-PA. For a comparative analysis of hirudin in various models, see Just et al. (45).

2.8. Foreign Surface-Induced Thrombosis

The presence of foreign materials in the circulation results in activation of the coagulation and platelet systems. A variety of prothrombotic surfaces have been used for the development of experimental animal thrombosis models. In contrast to many other thrombosis models, thrombosis induced by foreign surfaces does not presuppose endothelial damage.

2.8.1. Wire Coil-Induced Thrombosis

2.8.1.1. Purpose and Rationale

This classical method of producing thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (121) using the dog aorta and was further modified for use in arterial coronary vessels of opened-chest dogs. The formation of thrombotic material around
the coil is reproducible and can be easily standardized for pharmacological studies (48, 122, 123).

The use of this model in venous vessels was described by Kumada et al. (124). Venous thrombosis is produced in rats by insertion of a stainless steel wire coil into the inferior caval vein. Platelets and plasmatic coagulation are activated on the wire coil. Thrombus formation on the wire is quantitated by measuring the protein content of the isolated thrombotic material. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min of insertion, followed by a period of steady state flux between thrombus formation and endogenous thrombolysis, leading to a level protein content of thrombi starting at 1 h and lasting up to 48 h after implantation. The incidence of thrombosis in untreated control animals in this model is 100%. The model is used to evaluate antithrombotic and thrombolytic properties of test compounds in an in vivo model of venous thrombosis in rats.

**2.8.1.2. Procedure**

Male Sprague-Dawley rats weighing 260–300 g receive test compound, or vehicle as a control, by oral, i.v. or i.p. administration. At the end of absorption (i.v., 1 min; i.p., 30 min; p.o., 60 min), the animals are anesthetized by i.p. injection of 1.3 g/kg of urethane. Through a midline incision the caudal caval vein is exposed and a stainless steel wire coil (Zipperer®, size 40; Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting the wire toward the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured and the animal is placed on its back on a heating pad (37°C). The wound is reopened after 2 h and the wire coil with the thrombus on it is carefully removed and rinsed with a 0.9% saline solution. The thrombotic material is dissolved in 2 ml of alkaline sodium carbonate solution (2% Na₂CO₃ in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 μl aliquots by the colorimetric method of Lowry (Fig. 2.4).

**Thrombolysis.** In addition to the procedure described above, a thrombolytic test solution is continuously infused through a polyethylene catheter inserted into the jugular vein. Ninety minutes after wire implantation, the test compound or the vehicle (control) is infused for up to 2.5 h. The wire coil is then removed and the protein content of the thrombus is determined. Using this model, Bernat et al. (125) demonstrated the fibrinolytic activity of urokinase and SK–human plasminogen complex.

**2.8.1.3. Evaluation**

1. Thrombosis incidence = number of animals in each dosage group that develop thrombi as compared to the vehicle control.
2. The mean protein content [mg] of thrombotic material in each dosage group as compared to the vehicle control is determined. Percent change in protein content is calculated relative to control.

3. Statistical significance is assessed by means of the unpaired Student’s t-test.

2.8.2. Eversion Graft-Induced Thrombosis

2.8.2.1. Purpose and Rationale

The eversion graft model of thrombosis in the rabbit artery was first described by Hergrueter et al. (126) and later modified by
Jang et al. (127, 128) and Gold et al. (15). A 4- to 6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted, and then re-implanted into the vessel by end-to-end anastomosis. After restoration of blood flow, a platelet-rich occlusive thrombus forms rapidly leading to complete occlusion of the vessel. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction, which often occurs in the inverted femoral segments.

2.8.2.2. Procedure

In anaesthetized New Zealand white rabbits, the right carotid artery is exposed. After double ligation, a 3-mm segment of the artery is excised, everted, and immersed in pre-warmed (37°C) isotonic saline. The right femoral artery is exposed and occluded by means of a double occluder (2-cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures and 9-0 nylon (Prolene; Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650; Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flow meter (Model T106; Transonic, Ithaca, NY, USA). The flow probe is positioned 2 cm distal from the graft. After a stabilization period of 15 min, the test substance is administered i.v. through the catheterized right jugular vein. Ten minutes after administration of the test compound, the vessel clamps are released and blood flow is monitored by the flow meter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before administration of test compound), and 10, 60, and 120 min after administration.

2.8.2.3. Evaluation

1. Time until occlusion = time between restoration of vessel blood flow and occlusion of the vessel, as indicated by a flow of less than 3.0 ml/min.

2. Patency = time during which perfusion of the graft is measured relative to an observation period of 120 min after administration of test compound.

3. Time until occlusion and patency are expressed as median and inter-quartile range/2 (IQR/2). Significant differences ($P<0.05$) are calculated by the nonparametric Kruskal–Wallis test.

2.8.2.4. Critical Assessment of the Method

The eversion graft is very thrombogenic, although technically difficult and time consuming. The resultant deep occlusive thrombi can be prevented only by intra-arterial administration of thrombolytics or aggressive antithrombotic treatments, such as high doses of recombinant hirudin or PEG-hirudin. Because the initiating surface is a non-endothelial tissue containing tissue factor and collagen, both the coagulation and the blood platelet systems are activated.
Gold et al. (15) described a modification of this model in partially obstructed left circumflexed coronary arteries of thoracotomized dogs. The combination of reduced blood flow due to the constrictor and an abnormal non-endothelial surface produces total thrombotic occlusion within 5 min.

Models that use a catheter to induce vessel wall damage of both arteries and veins have been reported (63, 129–131). Such models in the arterial system mimic potential injuries induced by angioplasty. In these models, the endothelium is damaged either by rubbing the catheter across the luminal surface of the vessel or by air desiccation. Inflation of the balloon and the induction of partial stasis in the area of damage produce additional injury. By this procedure, vessel wall collagen, elastic tissues, and tissue thromboplastin are exposed to the circulating blood. Such models are typically carried out in rabbits or larger animals due to size considerations for both the vessel and the catheter.

In these models, the formation of thrombi has been detected in a number of ways. Measurement of flow by a distally placed flow meter has been reported (130). A decrease in vessel temperature measured distally to the site of injury is reflective of a decrease in blood flow through the segment and the formation of a thrombus. Deposition of radiolabeled platelets at the site of injury and measurement of thrombus wet weight have also been used.

Platelets appear to play an important role in the formation of thrombi at sites where the endothelium is damaged (132). Platelets may also play a key role in the initiation of the restenotic process following angioplasty (133). These models, therefore, provide the opportunity to assess the pharmacologic effects of agents capable of modulating either acute platelet function or the coagulation system that may be useful as adjunctive treatments in angioplasty. It has been demonstrated that both the platelet and the clotting systems are activated by arterial intervention (133, 134) and with this model, it has been shown that heparin and hirudin are both capable of inhibiting initial thrombosis. In addition, these models have also been used to assess the inhibition of re-thrombosis following lysis of the initial clot (130).

A method for the direct observation of extracorporeal thrombus formation was introduced by Rowntree and Shionoya (135). Very early studies using this model provided evidence that anticoagulants like heparin and hirudin inhibit thrombus development in AV shunts. Today, AV shunt thrombosis models are often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (136), rats
Rats are anaesthetized and fixed in a supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are connected with a 2-cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of test compound, the clamps that are occluding the AV shunt are opened.

Patency of the shunt is measured indirectly using a NiCrNi-thermocouple fixed distal to the glass capillary. When blood is flowing, the temperature rises from room temperature to body temperature. By comparison, decreased temperature indicates the formation of an occluding thrombus. Temperature is measured continuously over 30 min after the opening of the shunt.

It has been shown by Best et al. (139) that thrombi formed in the AV shunt are to a great extent white arterial thrombi. This might be due to the high pressure and shear rate inside the shunts, causing thrombi to be more arterial in character (14).

Compared to the arterial system, the development of thrombosis models in venous blood vessels tends to be more difficult in terms of reproducibility and variability (14). Complete stasis together with a thrombogenic stimulus (Wessler-type) has been used by a number of investigators to evaluate the effects of test compounds on venous thrombosis. Hollenbach et al. (141) developed a rabbit model of venous thrombosis by introducing cotton threads into the abdominal vena cava of rabbits. The cotton thread serves as a thrombogenic surface, and the thrombus that forms around it reaches a maximum mass after 2–3 h. The prolonged non-occlusive character of thrombogenesis in this model enables studies that focus on the progression of thrombus formation rather than initiation. Thus, conditions more closely resemble the pathophysiology of thrombosis in humans, because blood continues to flow throughout the experiment (14).
vena cava and vena iliac are dissected free from surrounding tissue. The test compound is administered by an intragastric tube for 60 min (depending on the results of ex vivo analysis) prior to initiation of thrombus formation. Blood samples are analyzed 60, 90, 120, 150, and 210 min after oral administration of the test compound.

Thrombus formation is induced by inserting the thrombosis catheter into the caval vein via the vena iliaca (7 cm). The copper wire is pushed forward 3 cm to release the cotton threads into the vessel lumen. After thrombus initiation (150 min after initiation), the caval segment containing the cotton threads and the developed thrombus is removed, opened longitudinally, and the content is blotted onto filter paper. After weighing the cotton threads with thrombus, the net dry thread weight is subtracted to determine the corrected thrombus weight.

2.8.4.3. Evaluation

1. Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net dry weight of the cotton thread.
2. Mean arterial blood pressure (MAP).
3. aPTT, HepTest, anti-FIIa, and anti-FXa activities.

2.8.4.4. Critical Assessment of the Method

The cotton thread-induced thrombus is composed of fibrin together with tightly aggregated and distorted erythrocytes, similar to human deep vein thrombosis structure. Non-occlusive thrombus formation in this model has been successfully inhibited by heparins, prothrombinase complex inhibitors, and thrombin inhibitors (141, 142).

2.8.4.5. Modifications of the Method

In addition to the originally described method, it is possible to measure blood flow by means of an ultrasonic flow probe attached distal to the position of the cotton threads on the vein.

2.8.5. Thrombus Formation on Superfused Tendon

2.8.5.1. Purpose and Rationale

In all models that include vessel wall damage, blood comes in contact with adhesive proteins of the sub-endothelial matrix, i.e., von Willebrand factor, collagen, fibronectin, laminin, and others. Gryglewski et al. (143) described an in vivo method in which the blood of a non-anesthetized animal is exposed ex vivo to a foreign surface consisting mainly of collagen. The foreign surface is a part of the tendon of another animal species. After superfusion of the tendon, blood is re-circulated to the non-anesthetized animal. This method enables the quantitation of antiplatelet potency based on the formation of platelet thrombi on the surface of the tendon or aortic strips from atherosclerotic rabbits.
2.8.5.2. Procedure

Blood is withdrawn from the carotid artery of anesthetized and heparinized cats using a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37°C), blood is separated into two streams, each flowing at a speed of 3 ml/min, that superfuse in parallel two twin strips of the central part of the longitudinally cut rabbit Achilles tendon (30×3 mm). After superfusing the tendon strips, the blood is allowed to drip into collectors and return to the venous system of the animal by gravity through the left jugular vein. The tendon strips are freely suspended in the air. The upper ends are tied to an auxotonic lever of a smooth muscle/heart Harvard transducer; the lower ends are loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips become covered with clots, changing the weight of the strips. Weight changes are continuously recorded. After a control period of 30 min, the formed thrombi are gently removed and fixed in formalin for histological examination. The strips are superfused with Tyrode solution, and the animals are injected with antithrombotic (test) compound. After 10 min, blood superfusion is repeated for another 30 min.

2.8.5.3. Evaluation

The ratio of weight increase of the strips after drug treatment relative to before drug treatment is taken as an index of antiaggregatory activity.

2.9. Stasis-Induced Thrombosis (Wessler Model)

2.9.1. Purpose and Rationale

The Wessler model is a classic method of inducing venous thrombosis in animals. Wessler (3, 144–148) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after injection of the procoagulatory stimulus into the circulation. Within a few minutes after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (149) summarizes the variety of substances that can be used as procoagulatory stimuli in this model. Aronson and Thomas (150) found an inverse correlation between the duration of stasis and the amount of hypercoagulation agent used to produce the clot.

2.9.2. Procedure

Anaesthetized rabbits are fixed in a supine position on a temperature-controlled (37°C) heating table. Following cannulation of both carotid arteries (the left in a cranial direction) and the right vena femoralis, segments (2 cm in length) of the two external jugular veins are exposed and isolated between
two loose sutures. Calcium thromboplastin (0.3 ml/kg) (Sigma; Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 s followed by injection of 0.5 ml of physiological saline within 15 s. Both jugular vein segments are occluded 45 s later by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 s before the end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by longitudinal incision.

2.9.3. Evaluation

The size of the clots is assessed using a scoring system: 0, blood only; 1, very small clot piece, filling up to 1/4 of the vessel; 2, larger clot, filling up to 1/2 of the vessel; 3, very large clot, filling up to 3/4 of the vessel; 4, a single, large clot that fills the whole vessel. The scores for the left and the right jugular veins are added to arrive at a thrombus size value for each animal. Thrombus weight is also measured after blotting the thrombus on filter paper.

Thrombus score is expressed as a median (minimum–maximum). Thrombus weight is expressed as mean ± SEM. For the statistical evaluation of antithrombotic effects, the nonparametric U-test of Mann and Whitney (thrombus score) or Student’s t-test for unpaired samples (thrombus weight) is used. Significance is expressed as P < 0.05.

2.9.4. Critical Assessment of the Method

Because of its static design, Breddin (151) described the Wessler model as the retransformation of an in vitro experiment into a very artificial test situation. One of the major drawbacks of the Wessler model is that it is relatively independent of platelet function and hemodynamic changes that largely influence thrombus formation in vivo. However, the model has been shown to be very useful for evaluating the antithrombotic effects of compounds like heparin and hirudin.

2.9.5. Modifications of the Method

There are a number of different procoagulant agents, such as human serum, Russel viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates, and FXa, that have been used to induce thrombosis in this model (149, 150). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring specific radioactivity of the clot.

A general drawback of the Wessler model is the static nature of venous thrombus development. To overcome this problem, some investigators have developed more dynamic models that incorporate reperfusion of the occluded vessel segments after clot development. Depending on the time of administration of test compound (pre- or post-thrombus initiation), the effect on
thrombus growth and fibrinolysis can be evaluated. Levi et al. (152) have used this model to assess the effects of a murine monoclonal anti-human PAI-1 antibody, and Biemond et al. (153) compared the effects of thrombin and FXa inhibitors to a low-molecular weight heparin using a modified Wessler model.

**Venous reperfusion model.** New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml of atropine, 1.0 mg/kg of diazepam, and 0.3 ml of Hypnorm (Duphar; 10 mg/ml fluanisone and 0.2 ml fentanyl). Anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposure through an incision in the neck. The jugular vein is dissected free from tissue and small side branches are ligated over a distance of 2 cm. The vein is clamped proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with $^{131}$I-fibrinogen (approximately 25 mCi/ml), and then 150 μl of radiolabeled blood is aspirated in a 1-ml syringe containing 25 μl of thrombin (3.75 IU) and 45 μl of 0.25 M CaCl$_2$. An aliquot (200 μl) of the clotting blood is immediately injected into the isolated segment. Thirty minutes after clot injection, the vessel clamps are removed and blood flow is restored. $^{125}$I-fibrinogen (approximately 5 μCi) is injected through the cannula in the carotid artery (in the case of fibrinolysis studies, this is immediately followed by injection of 0.5 mg/kg recombinant t-PA). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measuring $^{131}$I-fibrinogen remaining in the clot (relative to initial clot radioactivity). Comparison of $^{125}$I levels in blood and thrombus is a measure of the extent of thrombus growth. Thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as a percentage of the initial thrombus volume. Statistical analysis is carried out using variance analysis and the Newman–Keuls test. Statistical significance is expressed as $P < 0.05$.

**2.10. Disseminated Intravascular Coagulation (DIC) Model**

**2.10.1. Purpose and Rationale**

Widely used in rats and mice, the DIC model is a model of systemic thrombosis induced by tissue factor, endotoxin (lipopolysaccharide), or FXa (154–156). After systemic administration of a thrombogenic stimulus, studies can be performed with or without mechanical vena caval stasis. When stasis is used, the major parameter is thrombus mass; when stasis is not used, the parameters are primarily fibrin degradation products, fibrinogen, platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT). Given the many and varied
parameters that are measured when stenosis is not used, post-experimental analysis can be time consuming and technically demanding. Although rodents are useful as a primary efficacy model, limitations in drawing multiple blood samples over the course of the experiment and differences in activity of at least some FXa inhibitors in human as compared to rat plasma in vitro require that compounds be further characterized in more advanced in vivo models of thrombosis.

2.11. Microvascular Thrombosis in Trauma Models

2.11.1. Purpose and Rationale

Successful re-plantation of amputated extremities is dependent to a large degree on maintaining the microcirculation. A number of models have been developed in which blood vessels are subjected to crush injury with or without vascular avulsion and subsequent anastomosis (157–159). In the model of Stockmans (159), both femoral veins are dissected from the surrounding tissue. A trauma clamp, which has been adjusted to produce a pressure of 1,500 g/mm², is positioned parallel to the long axis of the vein. The anterior wall of the vessel is grasped between the walls of the trauma clamp and the two endothelial surfaces are rubbed together for a period of 30 s as the clamp is rotated. Formation and dissolution of platelet-rich mural thrombi are monitored over a period of 35 min by transillumination of the vessel. By using both femoral veins, the effect of drug therapy can be compared to control in the same animal, minimizing intra-animal variations.

The models of Korompilias (158) and Fu (157) examine the formation of arterial thrombosis in rats and rabbits, respectively. In these models, either the rat femoral artery or the rabbit central ear artery is subjected to a standardized crush injury. The vessels are subsequently divided at the midpoint of the crushed area and then anastomosed. Vessel patency is evaluated by milking the vessel at various time points post-anastomosis. These models have been used to demonstrate the effectiveness of topical administration of LMWH in preventing thrombotic occlusion of the vessels. Such models, while effectively mimicking the clinical situation, are limited by the necessity of a high degree of surgical skill to effectively anastomose the crushed arteries.

2.12. Cardiopulmonary Bypass Models

2.12.1. Purpose and Rationale

Cardiopulmonary bypass (CPB) models have been described in baboons (160), swine (161), and dogs (162). In each model, the variables that can affect the hemostatic system such as anesthesia,
shear stress caused by the CPB pumps, and the exposure of plasma components and blood cells to foreign surfaces (i.e. catheters, oxygenators, etc) are comparable to that observed with human patients. With these models, it is possible to examine the potential usefulness of novel anticoagulants in preventing thrombosis under relatively harsh conditions where both coagulation and platelet function are altered. The effectiveness of direct thrombin inhibitors (160), LMWHs (163), and heparinoids (162) has been compared to standard heparin using this model. Endpoints have included the measurement of plasma anticoagulant levels, histological determination of microthrombi deposition in various organs, formation of blood clots in components of the extracorporeal circuit, and the deposition of radiolabeled platelets in various organs and components of the extracorporeal circuit. These models, therefore, can be used to assess the antithrombotic potential of new agents for use in CPB surgery and also to assess the biocompatibility of components used to maintain extracorporeal circulation. The reader is referred to several detailed protocols and evaluations of this model (3–9, 157–159).

2.13. Extracorporeal Thrombosis Models

2.13.1. Purpose and Rationale

These models employ passing blood over a section of damaged vessel (or other selected substrate) and recording thrombus accumulation on the damaged vessel histologically or by scintigraphic detection of radiolabeled platelets or fibrin (164). This model is interesting because the results can be directly compared to in vivo deep arterial injury model (165) results and to results from a similar extracorporeal model used in humans (166, 167). Dangas et al. (166) used this model to characterize the antithrombotic efficacy of abciximab, a monoclonal antibody-based platelet GPIIb/IIIa inhibitor, after administration to patients undergoing percutaneous coronary intervention. They demonstrated that abciximab reduces both the platelet and the fibrin components of the thrombus, thereby providing further insight into the unique long-term effectiveness of short-term administration of this drug. Ørvim et al. (167) also used this model in humans to evaluate the antithrombotic efficacy of recombinant-tick anticoagulant peptide (rTAP), but instead of evaluating the compound after administration of rTAP to the patient, the drug was mixed with the blood immediately as it flowed into the extracorporeal circuit prior to flowing over the thrombogenic surface. By changing the thrombogenic surface, they were able to determine that rTAP was more effective at inhibiting thrombus formation on a tissue factor-coated surface compared to a collagen-coated surface. These results suggest that optimal antithrombotic efficacy requires an antiplatelet approach along with an anticoagulant.
Although this model does not completely represent pathological intravascular thrombus formation, the use of this “human model” of thrombosis may be very useful in developing new drugs because it directly evaluates the ex vivo antithrombotic effect of a drug in flowing human blood.

2.14. Experimental Thrombocytopenia or Leucocytopenia

2.14.1. Purpose and Rationale

Intravenous administration of collagen, arachidonic acid, ADP, PAF, or thrombin activates thrombocytes leading to maximal thrombocytopenia within a few minutes. This effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravascular aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of agonist, this experimentally-induced reduction in circulating platelets is reversible within 60 min after induction. Following administration of PAF (or other agonist), leucocytopenia is induced by the addition of epinephrine. This assay is used to test the inhibitory capacity of compounds against thrombocytopenia or leucocytopenia arising from in vivo platelet or leukocyte stimulation.

2.14.2. Procedure

Male guinea pigs (Pirbright White) (300–600 g), male NMRI mice (25–36 g), or Chinchilla rabbits of either sex (2–3 kg) are used. Animals receive test compound or vehicle as a control by oral, i.p., or i.v. administration (Table 2.1). After absorption time (p.o., 60 min; i.p., 30 min; i.v., variable), the marginal vein of the ear of rabbits is cannulated and a thrombocytopenia-inducing substance (i.e., collagen or arachidonic acid) is injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun® (i.m.) and placed on a temperature-controlled table at 37°C. The carotid artery is cannulated for blood withdrawal and the jugular vein is cannulated to administer thrombocytopenia-inducing substance(s), such as collagen+adrenaline, PAF, or thrombin (see Table 2.1). For mice, collagen+adrenaline is injected into a tail vein. Approximately 50–100 μl of blood is collected into potassium-EDTA-coated tubes 1 min before (−1), and 1 and 2 min after injection of the inducer (for guinea pigs and mice), or 5, 10, and 15 min (for rabbits) after inducer. The number of platelets and leukocytes is determined within 1 h of withdrawal in aliquots of 10 μl of whole blood using a microcell counter suitable for blood of various animal species.
Table 2.1
Experimental thrombocytopenia or leucocytopenia: materials and solutions

<table>
<thead>
<tr>
<th>Substance used to induce thrombocytopenia/leucocytopenia (i.v. administration)</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbits:</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (Sigma)</td>
<td>1 mg/kg</td>
</tr>
<tr>
<td>Collagen (Hormonchemie)</td>
<td>30 μg/ml</td>
</tr>
<tr>
<td>Mice:</td>
<td></td>
</tr>
<tr>
<td>Collagen + adrenaline (Hormonchemie)</td>
<td>90 μg/kg + 20 μg/kg</td>
</tr>
<tr>
<td>Hamsters:</td>
<td></td>
</tr>
<tr>
<td>Collagen + adrenaline</td>
<td>50 μg/kg + 10 μg/kg</td>
</tr>
<tr>
<td>Guinea pigs:</td>
<td></td>
</tr>
<tr>
<td>PAF (Paf-acether, Bachem)</td>
<td>0.03–0.04 μg/kg</td>
</tr>
<tr>
<td>Thrombin (Hoffman-LaRoche)</td>
<td>60 U/kg</td>
</tr>
<tr>
<td>Anesthetics:</td>
<td></td>
</tr>
<tr>
<td>Pentobarbital sodium (i.p.)</td>
<td>30 mg/kg</td>
</tr>
<tr>
<td>Xylazine (i.m.)</td>
<td>8 mg/kg</td>
</tr>
<tr>
<td>Urethane (i.p.)</td>
<td>1.5 g/kg</td>
</tr>
<tr>
<td>Platelet analyzer: Sysmex microcellcounter F-800</td>
<td></td>
</tr>
</tbody>
</table>

2.14.3. Evaluation

1. The percentage of thrombocytes (or leukocytes) in vehicle control and dosage groups at different time points after injection of the inducer is determined relative to the initial value (before injection). The values for the controls are set as 100%.

2. Percent inhibition of thrombocytopenia (or leucocytopenia) is calculated in each dosage group relative to the control.

3. Statistical significance is evaluated by means of the unpaired Student’s t-test.

2.14.4. Critical Assessment of the Method

The method of collagen + epinephrine-induced thrombocytopenia has been widely used to study the phenotypes of knock-out mice carrying deletions of specific genes implicated in hemostasis/thrombosis. Recent examples are the Gas 6−/− knockout mouse (168) and mice lacking the gene for the Gz small G protein (169). The advantages of this method are that it is a simple experimental procedure, and a small volume of blood is required. In general, application of the method in small animals (mice, hamsters) requires small amounts of test compound. This model is a useful first step in assessing the in vivo antithrombotic efficacy of antiplatelet drugs.
2.15. Collagenase-Induced Thrombocytopenia

2.15.1. Purpose and Rationale

Intravenous administration of the proteolytic enzyme collagenase leads to the formation of endothelial gaps and exposure of deeper layers of the vessel wall. This type of vascular endothelial injury mainly triggers thrombus formation through the activation of platelets by contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. This model is used as an alternative to the model described above to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats.

2.15.2. Procedure

Male Sprague-Dawley rats weighing 260–300 g are used. The animals receive test compound, or vehicle as a control, by oral, i.p., or i.v. administration. After absorption time (i.p., 30 min; p.o., 60 min; i.v., variable), rats are anesthetized with pentobarbital sodium (i.p.) (see Table 2.2). One carotid artery is cannulated for blood withdrawal and one jugular vein is cannulated for injection of inducer. The animals receive an i.v. injection of heparin, and then 20 min later, approximately 100 μl of blood is collected (initial value). Ten minutes later, a thrombocytopenia-inducing substance (collagenase) is administered intravenously.

Five, ten, twenty, and thirty minutes after the injection of collagenase, blood samples (approximately 100 μl each) are collected into potassium–EDTA-coated tubes. The number of platelets is determined in 10 μl aliquots of whole blood within 1 h after blood withdrawal using a microcell counter. For additional details of this method, see Volkl and Dierichs (170).

2.15.3. Evaluation

1. The percentage of platelets in vehicle control and dosage groups at the different times following injection of collagen-

| Table 2.2 |
| Collagenase-induced thrombocytopenia: materials and solutions |

<table>
<thead>
<tr>
<th>Material/Compound</th>
<th>Description/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anesthetic:</td>
<td>pentobarbital sodium (i.p.) 60 mg/kg</td>
</tr>
<tr>
<td>Heparin (Liquemin®) (i.v.)</td>
<td>500 U/kg</td>
</tr>
<tr>
<td>Thrombocytopenia induction: collagenase (i.v.) (E.C. 3.4.24.3; Boehringer Mannheim)</td>
<td>10 mg/ml/kg</td>
</tr>
<tr>
<td>Platelet analyzer: Sysmex microcellcounter F-800</td>
<td></td>
</tr>
</tbody>
</table>
ase is determined relative to the initial value for each group. The values for the controls are set as 100%.

2. Percent inhibition of thrombocytopenia is calculated in each dosage group relative to the control.

3. Statistical significance is evaluated by means of the unpaired Student’s $t$-test.

### 2.16. Reversible Intravital Aggregation of Platelets

#### 2.16.1. Purpose and Rationale

Isotopic labeling of platelets can be used to monitor platelet aggregation and desegregation in vivo. ADP, PAF, arachidonic acid, thrombin, and collagen are known to induce platelet aggregation. In this model, labeled platelets are continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation-promoting agents produces an increase in radioactivity in A and a decrease in radioactivity in B. This method assumes that platelets aggregate within the vascular system and accumulate in the pulmonary microvasculature. This in vivo method can be used to evaluate the platelet anti-aggregatory properties of test compounds.

#### 2.16.2. Procedure

##### 2.16.2.1. Preparation of Labeled Platelets

Blood is obtained from rats by cardiopuncture. After centrifugation at $240 \times g$ for 10 min, platelet-rich plasma (PRP) is transferred to a clean tube and re-suspended in calcium-free Tyrode solution containing 250 ng/ml prostaglandin E1 (PGE1). The suspension is subjected to centrifugation at $640 \times g$ for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking in calcium-free Tyrode solution containing 250 ng/ml PGE1. $^{51}$Cr is added to 1 ml of the platelet suspension. Following a 20-min incubation period at $37^\circ$C, the suspension is again subjected to centrifugation at $640 \times g$ for 10 min. The supernatant is removed and the labeled platelets are re-suspended in 1 ml of calcium-free Tyrode solution containing 250 ng/ml PGE1.

##### 2.16.2.2. Experimental Course

Male Sprague-Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anaesthetized with pentobarbital sodium (30 mg/kg, i.p.). Following tracheotomy, the vena femoralis is exposed and cannulated. The labeled platelets are administered via the cannula. Circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. Counts are collected using a dual channel gamma spectrometer (Nuclear Enterprise 4681) integrated with a microcomputer (AM 9080A). One hour after administration of
labeled platelets (or when counts in A and B have stabilized), an aggregation-promoting agent (i.e., ADP, PAF, arachidonic acid, thrombin, or collagen) is administered twice by i.v. injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 h after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (for ADP, PAF, arachidonic acid, or thrombin), or one hour (for collagen) after administration of test compound, another injection of aggregating agent is administered. This injection serves as an additional control or to determine the long-term efficacy of a test compound.

2.16.2.3. Standard Compound

PGI2 (prostacyclin).

2.16.3. Evaluation

1. The microcomputer continuously collects information about aggregation and desegregation of labeled platelets.

2. The following parameters are recorded:
   - A: counts over the thorax
   - B: counts over the abdomen
   - Difference: A–B
   - Ratio: A/B

3. The time course of response is represented by a curve. The area under the curve is calculated using a specific computer software program.

4. Statistical significance is calculated using the Student’s $t$-test.

2.16.4. Modification of the Method

Oyekan and Botting (171) described a method for monitoring platelet aggregation in vivo in rats using platelets labeled with indium$^{3+}$ oxine in which the increase in radioactivity in the lung after injection of ADP or collagen was recorded.

Smith et al. (172) continuously monitored the intra-thoracic content of intravenously injected $^{111}$indium-labeled platelets in anesthetized guinea pigs using a microcomputer-based system.

3. Animal Models of Bleeding

3.1. Subaqueous Tail Bleeding Time in Rodents

3.1.1. Purpose and Rationale

Blood vessel damage results in the formation of a hemostatic plug, a process that involves several different mechanisms, including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot. A diagnostic parameter for specifically assessing defects of the hemostatic
In Vivo Models for the Evaluation of Antithrombotics and Thrombolytics

system and the influence of drugs on hemostasis is the length of time that it takes for bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. Transection of the tail of a rodent was first established by Döttl and Ripke (173), and today it is commonly used in experimental pharmacology.

3.1.2. Procedure

Anaesthetized rats are fixed in a supine position on a temperature-controlled (37°C) heated table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, test compound is administered. After a defined latency period, the tail of the rat is transected with a razor blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37°C).

3.1.3. Evaluation

The time until bleeding stops is determined within a maximum observation time of 600 s.

3.1.4. Critical Assessment of the Method

There are numerous variables that can influence bleeding time measurements in the rodent, as discussed in detail by Dejana et al. (174), including the position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, and method of injury (i.e., Simplate method, transection). These variables contribute to differences in results reported for compounds like aspirin and heparin analyzed under different assay conditions (175, 176). Furthermore, it is impossible to transect exactly one blood vessel, because the transected tail region consists of a few major arteries and veins, which mutually interact.

3.2. Arterial Bleeding Time in Mesentery

3.2.1. Purpose and Rationale

Arterial bleeding is induced by micro-puncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in viable blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and fibrin formation. In this test, compounds can be evaluated for their ability to inhibit thrombus formation, and thus prolong arterial bleeding time. This test is used to assess agents that interfere with primary hemostasis in small arteries.

3.2.2. Procedure

Male Sprague-Dawley rats weighing 180–240 g receive test compound, or vehicle as a control, by oral, i.p., or i.v. administration. After absorption time (i.p., 30 min; p.o., 60 min; i.v., variable), animals are anesthetized by i.p. injection of 60 mg/kg pentobarbital sodium. Rats are placed on a temperature-controlled table at 37°C.
The abdomen is opened by a midline incision and the mesentery is lifted to display the mesenteric arteries. The mesentery is draped over a plastic plate and superfused continuously with Tyrode solution maintained at 37°C. Bleeding times are determined on small mesenteric arteries (125–250 μm external diameter) at the junction of the mesentery with the intestines. Adipose tissue surrounding the vessel is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge; 16×5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40×. The time in seconds is determined from the time of puncture until bleeding is arrested by the formation of a hemostatic plug.

3.2.3. Evaluation

1. Mean bleeding times [s] are determined for each dosage group (4–6 animals/group, 4–6 punctures per animal) and compared to the respective control.

2. Statistical significance assessed by means of the unpaired Student’s t-test.

3. Prolongation of bleeding time in each dosage group is calculated relative to the vehicle control.

For additional details on the method and its use in evaluating various mechanisms or agents, refer to Butler et al. (177), Dejana et al. (174), and Zawilska et al. (178).

3.3. Template Bleeding Time

3.3.1. Purpose and Rationale

Template bleeding time is used to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system by way of a standardized linear incision in the skin (for human). This method has been modified with the development of a spring-loaded cassette with two disposable blades (Simplate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility in the length and depth of dermal incisions. Forsythe and Willis (179) described a modification that enables the Simplate technique to be used to analyze bleeding time in the oral mucosa of dogs.

3.3.2. Procedure

The dog is positioned in sternal or lateral recumbence. A strip of gauze is tied around the mandible and maxilla as a muzzle. The template device is placed evenly against the buccal mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1; Fisher Scientific Co., Clifton, NJ) held directly below, but not touching the wound. The filter paper is changed every 15 s. The end point for each bleeding is determined when the filter paper no longer develops a red crescent.
3.3.3. Evaluation

The time from triggering the device until blood no longer appears on the filter paper is recorded as the bleeding time. The normal range is 2–4 min.

3.3.4. Critical Assessment of the Method

The template bleeding time varies considerably between laboratories, as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times have been recognized in dogs with thrombocytopenia, von Willebrand's disease and uremia, and in dogs treated with aspirin, anticoagulants, and dextran (179, 180). Brassard and Meyers (181) reported that buccal mucosa bleeding time is sensitive to platelet adhesion and aggregation deficits. Generally, the effects of antithrombotic drugs in bleeding time models in animals do not exactly predict bleeding risks in clinical situations. However, the models allow comparison between drugs with different actions (174, 182).

3.3.5. Modifications of the Method

The Simplate device can also be used to perform incisions in the shaved inner ear of rabbits, taking care to avoid major vessels. Normal bleeding time in anaesthetized rabbits is approximately 100 s (77±4 s, n=20) (Just et al. unpublished data).

Klement et al. (180) described an ear bleeding model in anaesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37°C. Five full-thickness cuts were made with a No. 11 Bard-Parker scalpel blade, avoiding major vessels, and the ear was immediately re-immersed in saline. At different times thereafter (5–30 min), aliquots of the saline solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (183). A guillotine-type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. Normal bleeding time in this model ranges from 2 to 8 min.

4. Genetic Models of Hemostasis and Thrombosis

4.1. Purpose and Rationale

Recent advances in genetics and molecular biology have provided tools that allow scientists to design genetically altered animals
that are deficient in specific proteins involved in thrombosis and hemostasis (so-called knock-outs or nulls) (184, 185). These animals have been extremely useful for identifying and validating novel targets for therapeutic intervention. That is, by examining the phenotype (e.g., spontaneous bleeding, platelet defects, prolonged bleeding after surgical incision, etc.) of a specific knock-out strain, scientists can identify the role of the deleted protein. If the phenotype is favorable (e.g., not lethal), pharmacological agents can be designed to mimic the knockout. More recently, novel genetic-based medical approaches have also benefited greatly from the availability of these models, as discussed below. The following section briefly summarizes some of the major findings in thrombosis and hemostasis using genetically altered mice and concludes with an example of how these models have been used in the drug discovery process.

The majority of gene knockouts result in mice that develop normally, are born in the expected Mendelian ratios, and are viable, as defined by the ability to survive to adulthood. Although seemingly normal, these knock-out mice exhibit alterations in hemostatic regulation, especially when challenged. Deletion of FVIII, FIX, von Willebrand factor and $\beta_3$-integrin (186–189) all result in mice that bleed upon surgical challenge, and despite some minor differences in bleeding susceptibility, these mouse knock-out models mirror the human disease states quite well (hemophilia A, hemophilia B, von Willebrand disease, and Glanzmann’s thrombasthenia, respectively). Deletion of some hemostatic factors results in fragile mice with severe deficiencies in their ability to regulate blood loss. Prenatally, these mice appear to develop normally, but they are unable to survive the perinatal period due to severe hemorrhaging, in most cases due to the trauma of birth.

Genetic knockouts have also been useful in dissecting the role of individual signaling proteins in platelet activation. Deletion of $\beta_3$-integrin (188) or of $G_{aq}$ (190) results in dramatic impairment of agonist-induced platelet aggregation. Alteration of the protein coding region in the $\beta_3$-integrin carboxy tail, $\beta_3$-DiY, at sites that are thought to be phosphorylated upon platelet activation also results in unstable platelet aggregation (191). Deletion of various receptors, such as thromboxane A2, P-selectin, P2Y1, and PAR-3, results in diminished responses to some agonists, while other platelet responses are intact (192–195). Deletion of PAR-3, another thrombin receptor in mice, had little effect on hemostasis, indicating the presence of yet another thrombin receptor in platelets, leading to the identification of PAR-4 (192).

Given that knockouts of prothrombotic factors yield mice with bleeding tendencies, it follows that deletion of factors in the fibrinolytic pathway results in increased thrombotic susceptibility in mice. Plasminogen (196, 197), t-PA, urokinase-type plasmino-
gen activator (u-PA), and combined t-PA/u-PA double knock-out (198) result in mice with impaired fibrinolysis, susceptibility to thrombosis, vascular occlusion, and tissue damage due to fibrin deposition. Interestingly, due to fibrin formation in the heart, these mice might serve as good models of myocardial infarction and heart failure caused by thrombosis (199). Intriguingly, mice deficient in PAI-1, the primary inhibitor of plasminogen activator, exhibit no spontaneous bleeding and a greater resistance to venous thrombosis due to a mild fibrinolytic state (200), which suggests that inhibition of PAI-1 might be a promising target for novel antithrombotic agents.

In addition to their role in the regulation of hemostasis, several targeted genes are important in embryonic development. For example, deletion of tissue factor (201–203), tissue factor pathway inhibitor (TFPI) (204), or thrombomodulin (205) results in an embryonic lethal phenotype. These and other (206, 207) hemostatic factors also appear to contribute to vascular integrity in the developing embryo. These data suggest that initiation of coagulation and generation of thrombin is important at a critical stage of embryonic development, yet other factors must contribute, since some of these embryos are able to progress and survive to birth.

Clearly, genetically altered mice have provided valuable insight into the roles of specific hemostatic factors in physiology and pathophysiology. The results of studies using knock-out mice have provided rationale and impetus for attacking certain targets pharmacologically. Knock-out mice have also provided excellent model systems for studying novel treatments for human diseases. For example, genetically altered animals have provided exceptional systems for the development of gene therapy for hemophilia. Specifically, deletion of FIX, generated by specific deletions in the FIX gene and its promoter, results in mice with a phenotype that mimics the human phenotype of hemophilia B (208). When these mice are treated by adenovirus-mediated transfer of human FIX, the bleeding diathesis is fully corrected (209). Similarly, selectively bred dogs that have a characteristic point mutation in the gene sequence encoding the catalytic domain of FIX also have a severe hemophilia B that is phenotypically similar to the human disease (210). Adeno-associated virus-mediated delivery of the canine FIX gene to these dogs intramuscularly resulted in measurable, therapeutic levels of FIX for up to 17 months (211). Clinically relevant partial recovery of whole blood clotting time and aPTT was also observed over this prolonged period. These data provided support for the first study of adeno-associated virus-mediated FIX gene transfer in humans (212). Preliminary results from clinical studies showed evidence of expression of FIX in three hemophilia patients and also provided favorable safety data to substantiate the use of this
therapeutic at higher doses. Although it is likely that there are differences between the human disease and animal models of hemophilia (or other diseases), it is clear that these types of experiments can provide pharmacological, pharmacokinetic, and safety data that will be extremely useful in designing and developing approaches for safe clinical trials.

Gene therapy for patients with bleeding diatheses is more advanced than gene therapy for thrombotic indications. However, promising preclinical data indicates that local overexpression of thrombomodulin (213) or t-PA (214) inhibits thrombus formation in a rabbit model of arterial thrombosis. Similarly, local gene transfer of TFPI prevented thrombus formation in balloon-injured porcine carotid arteries (215). These and other studies (216) suggest that novel gene therapy approaches will also be effective for thrombotic indications, but these treatments will need to be carefully optimized in terms of pharmacokinetics, safety, and efficacy in laboratory animal studies prior to testing in humans.

4.1.1. Knock-out Mice

4.1.1.1. Factor I (Fibrinogen)
Mice are normal in appearance at birth. Approximately 10% die shortly after birth, and another 40% at 1–2 months after birth due to bleeding and/or failure of pregnancy. Blood fails to clot or support platelet aggregation in vitro (217).

4.1.1.2. Factor II (Prothrombin)
Factor II deletion is a partial embryonic lethal mutation, with a 50% rate of death between embryonic day (E) 9.5 and E11.5. At least 25% survive to term, but suffer from fatal hemorrhage a few days after birth. Factor II is important for maintaining vascular integrity during development and post-natally (218, 219).

4.1.1.3. Factor V
Half of the embryos die at E9–E10, possibly as a result of abnormal yolk sac vasculature. The remaining 50% progress normally to term, but die from massive hemorrhage within 2 h of birth. This defect results in a more severe phenotype in the mouse than in human (207, 218).

4.1.1.4. Factor VII
Mice develop normally but suffer fatal perinatal bleeding (219).

4.1.1.5. Factor VIII
Mice exhibit a mild phenotype as compared to severe hemophilia A in humans. There is no spontaneous bleeding, illness or reduced activity during the first year of life. Blood exhibits residual clotting activity (aPTT) (220).

4.1.1.6. Factor IX
Factor IX coagulant activity (aPTT) associated with wild-type, heterozygous, and homozygous null mice is as follows: +/+ , 92%; +/− , 53%; −/− , <5%, respectively. Mice suffer from a bleeding disorder, which has been characterized as extensive bleeding
after clipping a portion of the tail, with bleeding to death if not
cauterized (189, 221).

4.1.1.7. Factor X
Partial embryonic lethal (1/3 of the mice died on E11.5 or
E12.5), with fatal neonatal bleeding between perinatal day 5 (P5)
and P20 (222).

4.1.1.8. Factor XI
APTT is prolonged in homozygous null (−/−) mice (158–200 s)
as compared to wild-type (+/+, 25–34 s) and heterozygous (+/−,
40–61 s) mice. No factor XI activity; knockout does not result
in intrauterine death; homozygous null exhibit similar bleed-
ing times as wild-type mice, with a tendency to prolongation
(223).

4.1.1.9. TF (Tissue Factor)
Abnormal circulation from yolk sac to embryo at approximately
E8.5, leading to embryo wasting and death, most likely reflect-
ing the role of TF in blood vessel development (201–203,
224).

4.1.1.10. TFPI
Lethal, with no survivors beyond the neonatal period. Approxi-
mately 60% of mice die between E9.5 and E11.5, with signs of
yolk sac hemorrhage (204).

4.1.1.11. Thrombin Receptor (TR)
Approximately 50% of the mice die at E9–E10. Fifty percent
survive and become normal adult mice at the gross level, with no
bleeding diathesis. Null platelets strongly respond to thrombin,
whereas null fibroblasts loose their ability to respond to thrombin,
indicating the existence of a second TR (206, 225).

4.1.1.12. Thrombomodulin
The null mutation is an embryonic lethal, with embryos
dying before the development of a functional cardiovascu-
lar system. Mice die before E9.5 due to growth retardation.
Heterozygous mice develop normally without thrombotic com-
plications (199, 205, 226, 227).

4.1.1.13. Protein C
Mice appear to develop normally at the macroscopic level, but
exhibit obvious signs of bleeding and thrombosis. Mice do not
survive beyond 24 h after delivery. Microvascular thrombosis in
the brain and necrosis in the liver is observed. Plasma clottable
fibrinogen is undetectable, suggesting fibrinogen depletion and
secondary consumptive coagulopathy (228).

4.1.1.14. Plasminogen
Mice exhibit severe spontaneous thrombosis, reduced ovula-
tion and fertility, cachexia and short survival, severe glomeru-
lonephritis, impaired skin healing, and reduced macrophage and
keratinocyte migration (196, 197).

4.1.1.15. Alpha2-antiplasmin
Mice exhibit normal fertility, viability, and development; no
bleeding disorders; spontaneous lysis of injected clots, which is
indicative of enhanced fibrinolytic potential; and a significant
Mousa

reduction of renal fibrin deposition after lipopolysaccharide (LPS) administration (229).

4.1.1.16. t-PA

Mice exhibit extensive spontaneous fibrin deposition, severe spontaneous thrombosis, impaired neointima formation, reduced ovulation and fertility, cachexia and short survival, severe glomerulonephritis, and abnormal tissue remodeling (198, 199).

4.1.1.17. PAI-1

Mice exhibit reduced thrombotic incidence, no bleeding, accelerated neointima formation, reduced lung inflammation and reduced atherosclerosis. Detailed studies of PAI-1 knock-out mice have been reported by Carmeliet et al. (200), Eitzman et al. (230), Erickson et al. (231), Kawasaki et al. (212), and Pinsky et al. (232).

4.1.1.18. Vitronectin

Mice exhibit normal development, fertility, and survival; serum is completely deficient in “serum spreading factor” and PAI-1 binding activity. Mice also exhibit delayed arterial and venous thrombus formation (233, 234).

4.1.1.19. Urokinase, u-PA (Urinary-Type Plasminogen Activator)

Single u-PA knock-out mice are viable, fertile, and have a normal life span. Mice occasionally exhibit spontaneous fibrin deposition in normal and inflamed tissue and have a higher incidence of endotoxin-induced thrombosis. Combined t-PA/u-PA-knock-out mice survive embryonic development, but exhibit retarded growth, reduced fertility, and shortened life span; spontaneous fibrin deposits are more extensive and occur in more organs (198, 235).

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intra-abdominal bleeding that is directly related to transgene expression in the liver and elevated plasma u-PA levels. Approximately 50% of the transgenic mice die between 3 and 84 h after birth with severe hypofibrinogenemia and loss of clotting function.

4.1.1.20. UPAR (Urinary-Type Plasminogen Activator Receptor)

Mice are phenotypically normal with attenuated thrombocytopenia and mortality associated with severe malaria (236–239).

4.1.1.21. Gas 6 (Growth Arrest-Specific Gene 6 Product)

Mice are viable, fertile, and appear normal; they do not suffer spontaneous bleeding or thrombosis and have normal tail bleeding times. Platelets fail to aggregate irreversibly to ADP, collagen, or U 46619. Arterial and venous thrombosis is inhibited and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine (168).

4.1.1.22. GPIbα (Glycoprotein Ibα, Part of the GPIb-V–IX Complex)

Mice exhibit bleeding, thrombocytopenia and giant platelets (similar to human Bernard Soulier syndrome) (240).
### 4.1.1.23. GPV (Glycoprotein V, Part of the GPIb-V–IX Complex)

Mice exhibit increased thrombin responsiveness; GPV null platelets are normal in size; mice have normal amounts of GP Ib-IX and functional von Willebrand factor (vWF) binding. Null platelets are hyper-responsive to thrombin and have an increased aggregation response and shorter bleeding time, reflecting the activity of GPV as a negative modulator of platelet function (241).

### 4.1.1.24. GPIIb (Integrin Alpha IIb, Glycoprotein IIb, Part of the GPIIb–IIIa Complex)

Mice have a bleeding disorder similar to Glanzmann thrombasthenia in man. Null platelets fail to bind fibrinogen, aggregate and retract a fibrinogen clot; platelet granules do not contain fibrinogen (242).

### 4.1.1.25. GPIIIa (Integrin Beta3, Glycoprotein IIIa, Part of the GPIIb–IIIa Complex)

Mice are viable and fertile, but have increased fetal mortality. Mice exhibit features of Glanzmann thrombasthenia in man, i.e., defective platelet aggregation and clot retraction, spontaneous bleeding, prolonged bleeding times, dysfunctional osteoclasts, and the development of osteosclerosis with age (188, 243).

### 4.1.1.26. GPIIa (Glycoprotein IIa, Integrin β1, Part of the GPIa–IIa Complex)

Integrin β1 null platelets from conditional knock-out mice develop normally, and platelet count is normal; collagen-induced platelet aggregation is delayed but otherwise normal; the tyrosine phosphorylation pattern is normal but phosphorylation is delayed. The bleeding time in the bone marrow of chimeric mice is normal, and there are no major in vivo defects (244).

### 4.1.1.27. vWF

FVIII levels are strongly reduced due to defective protection by vWF. Mice exhibit highly prolonged bleeding times, hemorrhaging, and spontaneous bleeding. vWF knockout mice have been useful for investigating the role of vWF. Mice exhibit delayed platelet adhesion in ferric chloride-induced arteriolar injury (187, 245).

### 4.1.1.28. Thromboxane A2 Receptor (TXA2R)

Mice exhibit a mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid (195).

### 4.1.1.29. Prostacyclin Receptor (PGI2R)

Mice are viable, fertile, and normotensive, with increased susceptibility to thrombosis and reduced inflammatory and pain responses (246).

### 4.1.1.30. PECAM (Platelet: Endothelial Cell Adhesion Molecule)

Mice exhibit normal platelet aggregation; Duncan et al. (247) and Mahooti et al. (248) described prolonged bleeding times in PECAM knockout mice.

### 4.1.1.31. Pallid (Pa)

The pallid mouse, one of 13 hypo-pigment mouse mutants with a storage pool deficiency, is a model of human Herman sky Pudlak syndrome (the beige mouse is a model of Chediak Higashi syndrome). Pallid mice exhibit prolonged bleeding times, pigment dilution, elevated kidney lysosomal enzyme, serum α1 antitrypsin deficiency and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino
acid protein pallidin, which interacts with syntaxin 13, a protein that mediates vesicle docking and fusion (249).

Blood from knock-out mice exhibits defective aggregation in response to ADP, TXA2, thrombin, and collagen; shape change is normal (190, 250).

Mice exhibit impaired platelet aggregation in response to epinephrine, resistance to fatal thromboembolism, and exaggerated responses to cocaine. Morphine and antidepressant drugs have a reduced effect in knock-out mice (169).

Mice are viable and fertile, with decreased numbers of mature B cells, defective B cell and mast cell function, and defective Fcy receptor signaling, resulting in a loss of collagen-induced platelet aggregation (251).

Mice are viable and fertile, with prolonged bleeding times but minimally perturbed coagulation parameters. Mice exhibit reduced platelet interaction with injured mesenteric vasculature in vivo; failure of platelets to aggregate in response to standard agonists in vitro is associated with purinergic P2Y1 receptor desensitization and fibrin deposition at multiple organ sites (252).

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Mice are viable and fertile, with prolonged bleeding times but minimally perturbed coagulation parameters. Mice exhibit reduced platelet interaction with injured mesenteric vasculature in vivo; failure of platelets to aggregate in response to standard agonists in vitro is associated with purinergic P2Y1 receptor desensitization and fibrin deposition at multiple organ sites (252).

Mice are viable and fertile, unresponsive to cGMP and NO, and defective in vasodilator-stimulated phosphoprotein (VASP) phosphorylation. Mice exhibit increased adhesion and aggregation of platelets in vivo in ischemic/re-perfused mesenteric microcirculation and no compensation by the cAMP kinase system (253).

Mice are viable and fertile and exhibit mild platelet dysfunction with megakaryocyte hyperplasia, increased collagen/thrombin activation, and impaired cyclic nucleotide-mediated inhibition of platelet activation (254, 255).

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in ex vivo assays, and increased mortality in an ADP-induced mouse model of thromboembolism (256, 257).

Mice develop normally and are healthy. They exhibit no differences in reaction to endotoxin shock as compared to wild-type mice; however, they are resistant to the lethal effects of shock induced by PAF. Inflammation induced by arachidonic acid in these mice is markedly reduced (256).

Thrombopoietin-null and thrombopoietin receptor (c-Mpl)-null mice exhibit a 90% reduction in megakaryocyte and platelet levels.
However, despite these low platelet levels, mice do not exhibit excessive bleeding. Platelets that are present are morphologically normal and functional, indicating that in vivo, thrombopoietin is required for control of megakaryocyte and platelet number, but not maturation (258).

4.1.1.41.
Thrombospondin-1

Mice exhibit normal thrombin-induced platelet aggregation and higher numbers of circulating white blood cells.

Table 2.3
Genetic models of thrombosis and hemostasis

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Viable</th>
<th>Embryonic development/survival</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coagulation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein C</td>
<td>No</td>
<td>Normal/perinatal death</td>
<td>(228)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Yes</td>
<td>Normal/perinatal death</td>
<td>(217)</td>
</tr>
<tr>
<td>Fibrinogen-QAGVD</td>
<td>Yes</td>
<td>Normal</td>
<td>(217)</td>
</tr>
<tr>
<td>FV</td>
<td>No</td>
<td>Partial embryonic loss/perinatal death</td>
<td>(207)</td>
</tr>
<tr>
<td>FVII</td>
<td>Yes</td>
<td>Normal/perinatal death</td>
<td>(219)</td>
</tr>
<tr>
<td>FVIII</td>
<td>Yes</td>
<td>Normal</td>
<td>(186)</td>
</tr>
<tr>
<td>FIX</td>
<td>Yes</td>
<td>Normal</td>
<td>(189)</td>
</tr>
<tr>
<td>FXI</td>
<td>Yes</td>
<td>Normal</td>
<td>(223)</td>
</tr>
<tr>
<td>Tissue factor</td>
<td>No</td>
<td>Lethal</td>
<td>(201, 203)</td>
</tr>
<tr>
<td>TFPI</td>
<td>No</td>
<td>Lethal</td>
<td>(204)</td>
</tr>
<tr>
<td>vWF</td>
<td>Yes</td>
<td>Normal</td>
<td>(187)</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>No</td>
<td>Partial embryonic loss/perinatal death</td>
<td>(277, 278)</td>
</tr>
<tr>
<td><strong>Fibrinolytic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>u-PA and t-PA</td>
<td>Yes</td>
<td>Normal/growth retardation</td>
<td>(198)</td>
</tr>
<tr>
<td>uPAR</td>
<td>Yes</td>
<td>Normal</td>
<td>(237, 238)</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Yes</td>
<td>Normal/growth retardation</td>
<td>(196, 197)</td>
</tr>
<tr>
<td>PAI</td>
<td>Yes</td>
<td>Normal</td>
<td>(200)</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>No</td>
<td>Lethal</td>
<td>(205)</td>
</tr>
<tr>
<td><strong>Platelet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>Yes</td>
<td>Normal/partial embryonic loss</td>
<td>(188)</td>
</tr>
<tr>
<td>B3-DiYF</td>
<td>Yes</td>
<td>Normal</td>
<td>(191)</td>
</tr>
<tr>
<td>P-Selectin</td>
<td>Yes</td>
<td>Normal</td>
<td>(194)</td>
</tr>
<tr>
<td>PAR-1</td>
<td>Yes</td>
<td>Normal</td>
<td>(206)</td>
</tr>
<tr>
<td>PAR-3</td>
<td>Yes</td>
<td>Normal</td>
<td>(192)</td>
</tr>
<tr>
<td>Gαq</td>
<td>Yes</td>
<td>Normal/perinatal death</td>
<td>(190)</td>
</tr>
<tr>
<td>TXA2 receptor</td>
<td>Yes</td>
<td>Normal</td>
<td>(195)</td>
</tr>
<tr>
<td>P2Y1</td>
<td>Yes</td>
<td>Normal</td>
<td>(193)</td>
</tr>
</tbody>
</table>
Thrombospondin-1 has been implicated in normal lung homeostasis (258).

Mouse knock-out models of virtually all of the known hemostatic factors have been reported, as shown in Table 2.3.

5. Critical Issues in Experimental models

5.1. The Use of Positive Controls

Clearly, there are many antithrombotic agents that can be used to compare and contrast the antithrombotic efficacy and safety of novel agents. The classic antithrombotic agents are heparin, warfarin, and aspirin. However, new, more selective agents such as hirudin, LMWHs, and clopidogrel are commercially available that will either replace or augment these older treatments. Novel antithrombotic agents should certainly be demanded to demonstrate better efficacy than currently available therapy in animal models of thrombosis. This should be demonstrated by performing dose–response experiments that include maximally effective doses of each compound in the model. At the maximally effective dose, parameters such as aPTT, PT, template bleeding time, or other, more sensitive measurements of systemic hypocoagulability or bleeding should be compared. A good example of this approach is a study by Schumacher et al. (259), who compared the antithrombotic efficacy of argatroban and deltaparin in arterial and venous models of thrombosis. Consideration of potency and safety compared to other agents should be taken into account when advancing a drug through the testing funnel.

The early in vivo evaluation of compounds that demonstrate acceptable in vitro potency and selectivity requires evaluation of each compound alone in order to demonstrate antithrombotic efficacy. The antithrombotic landscape is becoming complicated by so many agents from which to choose that it will become increasingly difficult to design preclinical experiments that mimic the clinical setting in which poly-antithrombotic therapy is required for optimal efficacy and safety. Consequently, secondary and tertiary preclinical experiments will need to be carefully designed in order to answer these specific, important questions.

5.2. Evaluation of Bleeding Tendency

Although the clinical relevance of animal models of thrombosis has been well-established in terms of efficacy, the preclinical tests for evaluating safety, i.e., bleeding tendency, have not been as predictable. The difficulty in predicting major bleeding, such as intracranial hemorrhage, resulting from antithrombotic
or thrombolytic therapy stems from the complexity and lack of understanding of the mechanisms involved in this disorder. Predictors of anticoagulant-related intracranial hemorrhage are advanced age, hypertension, intensity and duration of treatment, head trauma, and prior neurologic disease (260, 261). These risk factors are clearly difficult, if not impossible, to simulate in laboratory animals. Consequently, more general tests of anticoagulation and primary hemostasis have been employed.

Coagulation assays provide an index of the systemic hypocoagulability of the blood after administration of antithrombotic agents; however, as indicated earlier, the sensitivity and specificity of these assays vary from compound-to-compound, so these assays do not provide a consistent safety measure across all mechanisms of inhibition. Consequently, many laboratories have attempted to develop procedures that provide an indication of bleeding risk by evaluating primary hemostasis after generating controlled incisions in anesthetized animals. Some of the tests used in evaluating FXa inhibitors include template bleeding time, tail transection bleeding time, cuticle bleeding time, and evaluation of clinical parameters such as hemoglobin and hematocrit. Unfortunately, template bleeding tests, even when performed in humans, have not been good predictors of major bleeding events in clinical trials (262–264). However, these tests have been able to demonstrate relative advantages of certain mechanisms and agents over others. For example, hirudin, a direct thrombin inhibitor, appears to have a narrow therapeutic window when used as an adjunct to thrombolysis in clinical trials, producing unacceptable major bleeding when administered at 0.6 mg/kg i.v. bolus, plus 0.2 mg/kg/h (265, 266). When the dose of hirudin was adjusted to avoid major bleeding (0.1 mg/kg and 0.1 mg/kg/h), no significant therapeutic advantage over heparin was observed. If the relative improvement in the ratio between efficacy and bleeding observed preclinically with FXa inhibitors compared to thrombin inhibitors such as hirudin is supported in future clinical trials, this will establish an important safety advantage for FXa inhibitors and provide valuable information for evaluating the safety of new antithrombotic agents in preclinical experiments.

5.3. Selection of Models Based on Species-Dependent Pharmacology/Physiology

As alluded to earlier, species selection for animal models of disease is often limited by the unique physiology of a particular disease target in different species or by the species specificity of the pharmacological agent for the target. For example, it was discovered relatively early in the development of platelet GPIIb/IIIa antagonists that these compounds were of limited use in rats (267) and that there was a dramatic species-dependent variation in the response of platelets to GPIIb/IIIa antagonists (268–270). This discovery led to the widespread use of larger animals (particularly dogs, whose platelet response to GPIIb/IIIa
antagonists resembles humans) in the evaluation of GPIIb/IIIa antagonists. Of course the larger animals required more compound for evaluation, which created a resource problem for medicinal chemists. This was especially problematic for companies that generated compounds by combinatorial parallel synthetic chemistry in which many compounds can be made, but usually in very small quantities. However, some pharmacologists devised clever experiments that partially overcame this problem. Cook et al. (271) administered a GPIIb/IIIa antagonist orally and intravenously to rats and then mixed platelet-rich plasma from the treated rats with platelet-rich plasma from untreated dogs. The mixture was then evaluated in an agonist-induced platelet aggregation assay and the resulting inhibition of canine platelet aggregation (rat platelets were relatively unresponsive to this GPIIb/IIIa antagonist) was due to the drug present in the plasma obtained from the rat. Using this method, only a small amount of drug is required to determine the relative bioavailability in rats. However, the animal models chosen for efficacy in that report (guinea pigs and dogs) were selected based on their favorable platelet response to the GPIIb/IIIa antagonist.

Similarly for inhibitors of FXa, there are significant variations in the activity of certain compounds against FXa purified from plasma of different species and in plasma-based clotting assays using plasma from different species. DX-9065 is much more potent against human FXa (Ki = 78 nM) than against rabbit (Ki = 102 nM) and rat (Ki = 1980 nM) FXa. Likewise, in the PT assay, DX-9065a was very potent in human plasma (concentration required to double PT, PT × 2, was 0.52 μM) and in squirrel monkey plasma (PT × 2 = 0.46 μM), but was much less potent in rabbit, dog, and rat plasma (PT × 2 = 1.5, 6.5, and 22.2 μM, respectively). Other FXa inhibitors have also demonstrated these species-dependent differences in activity (272–274). Regardless, the investigator must be aware of these differences so that appropriate human doses can be extrapolated from the laboratory animal studies.

Although in many cases the exact mechanism for the species-dependent differences in response to certain therapeutic agents remains unclear, these differences must be examined to determine the appropriate species to be used for preclinical pharmacological evaluation of each agent. This evaluation can routinely be performed by in vitro coagulation or platelet aggregation tests prior to evaluation in animal models.

5.4. Selection of Models Based on Pharmacokinetics

Much debate surrounds the issue as to which species most resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single
<table>
<thead>
<tr>
<th>Compound</th>
<th>Preclinical animal model</th>
<th>Preclinical results</th>
<th>Reference</th>
<th>Clinical indication</th>
<th>Clinical result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant t-PA</td>
<td>Rabbit pulmonary artery thrombosis</td>
<td>Lysis of preformed pulmonary thrombus</td>
<td>(279)</td>
<td>Acute myocardial infarction–thrombolysis</td>
<td>Improved recanalization</td>
<td>(280)</td>
</tr>
<tr>
<td>(Activase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abciximab</td>
<td>Canine coronary cyclic flow reduction (44)</td>
<td>Significant inhibition of platelet-dependent thrombosis</td>
<td>(39)</td>
<td>High-risk coronary angioplasty</td>
<td>Reduction in death, myocardial infarction, refractory ischemia, or unplanned revascularization</td>
<td>(281)</td>
</tr>
<tr>
<td>(ReoPro)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tirofiban</td>
<td>Canine coronary cyclic flow reduction (44)</td>
<td>Significant inhibition of platelet-dependent thrombosis</td>
<td>(282)</td>
<td>Unstable angina</td>
<td>Reduction in death, myocardial infarction, refractory ischemia</td>
<td>(283)</td>
</tr>
<tr>
<td>(Aggrestat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Eptifibatide</td>
<td>TPA-induced coronary thrombolysis</td>
<td>Significant improvement in lysis of occlusive thrombus</td>
<td>(81)</td>
<td>Acute myocardial infarction–thrombolysis with t-PA</td>
<td>Improvement in incidence and speed of reperfusion</td>
<td>(284)</td>
</tr>
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<td>(Integrilin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enoxaparin</td>
<td>Canine coronary cyclic flow reduction (44)</td>
<td>Significant inhibition of platelet-dependent thrombosis</td>
<td>(50)</td>
<td>Unstable angina</td>
<td>Significant decrease in death, myocardial infarction, and need for revascularization at 30 days</td>
<td>(285)</td>
</tr>
<tr>
<td>(Lovenox)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hirudin</td>
<td>Rabbit jugular vein thrombus growth</td>
<td>Inhibition of thrombus growth compared to standard heparin</td>
<td>(286)</td>
<td>Deep vein thrombosis after total hip replacement</td>
<td>Significantly decreased rate of DVT</td>
<td>(287)</td>
</tr>
<tr>
<td>(Refludan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Argatroban</td>
<td>Canine coronary artery electrolytic injury (TPA–induced thrombolysis)</td>
<td>Accelerated reperfusion and prevented re-occlusion</td>
<td>(288)</td>
<td>Unstable angina</td>
<td>No episodes of MI during drug infusion</td>
<td>(289)</td>
</tr>
</tbody>
</table>

Table 2.4
Animal models of thrombosis and their clinical correlates
animal can precisely mimic the gastrointestinal characteristics of humans (275). Due to resource issues (mainly compound availability) and animal care and use considerations, small rodents such as rats are usually considered for primary in vivo evaluation of pharmacokinetics for novel agents. However, there is great reservation about moving a compound into clinical trials based on oral bioavailability data derived from rat experiments alone. Usually, larger animals such as dogs or non-human primates, which have similar gastrointestinal morphology compared to humans, are the next step in the evaluation of pharmacokinetics of new agents. The pharmacokinetic characteristics of the FXa inhibitor YM-60828 have been studied extensively in a variety of laboratory animals. YM-60828 demonstrated species-dependent pharmacokinetics, with oral bioavailability estimates of approximately 4, 33, 7, and 20% in rats, guinea pigs, beagle dogs, and squirrel monkeys, respectively. Although these results suggest that YM-60828 has somewhat limited bioavailability, evaluating the pharmacokinetic profile of novel agents in a number of species (276) is a well-established approach used to aid in identifying compounds for advancement to human testing. That is, acceptable bioavailability in a number of species suggests that a compound will be bioavailable in humans. Which of the laboratory species adequately represents the bioavailability of a specific compound in humans can only be determined after appropriate pharmacokinetic evaluation in humans. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate animal model for testing the antithrombotic efficacy of compounds because the ultimate proof-of-concept experiment is to demonstrate efficacy by the intended route of administration.

6. Clinical Relevance of Data Derived from Experimental Models

Animal models of thrombosis have played a crucial role in the discovery and development of a number of compounds that are now successfully being used for the treatment and prevention of thrombotic diseases. Influential preclinical results using novel antithrombotics in a variety of laboratory animal experiments are listed in Table 2.4, along with the early clinical trials and results for each compound. This table intentionally omits many compounds that were tested in animal models of thrombosis, but failed to be successful in clinical trials or, for other reasons, did not become approved drugs. However, these negative outcomes would not have been predicted by animal models of thrombosis because the failures were generally due to other shortcomings of the drugs (e.g., toxicity, narrow therapeutic window, or
undesirable pharmacokinetics or pharmacodynamics) which are not always clearly presented in the scientific literature due to proprietary restrictions in this highly competitive field.

Nonetheless, it is clear that animal models have supplied valuable information for investigators responsible for evaluating these drugs in humans, providing pharmacodynamic, pharmacokinetic, and safety data that can be used to design safe and efficient clinical trials. The reader is referred to a number of detailed reports on the applications of animal models (188, 190–195, 197–213, 215, 216, 237).

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