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# Antithrombotic activity of a monoclonal antibody inducing the substrate form of plasminogen activator inhibitor type 1 in rat models of venous and arterial thrombosis

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**1** Elevated plasminogen activator inhibitor 1 (PAI-1) is a risk factor for thrombosis, and inhibitors of the interaction between PAI-1 and tissue plasminogen activator (t-PA) have antithrombotic and prothrombolytic activity in animals. We describe the antithrombotic effects in the rat of a monoclonal antibody (MA33H1) which converts PAI-1 to a non-inhibitory substrate.

**2** The activity of MA33H1 against rat PAI-1 was confirmed using two-chain t-PA and a chromogenic substrate. MA33H1 was evaluated in rat venous (thromboplastin + stasis in the abdominal vena cava) and arterial (electric current applied to a carotid artery) thrombosis models. The effects on tail-transection bleeding time were studied.

**3** MA33H1 at 100 ng $ml^{-1}$ inhibited both human (44.1%) and rat PAI-1 (49.7%). This effect was concentration-dependent. Its effect on human PAI-1 was not significantly inhibited by 1 $\mu$g $ml^{-1}$ fibrin or a $\approx$7 fold molar excess of vitronectin (1 nM). Inhibition of rat PAI-1 was unchanged by fibrin, but vitronectin reduced inhibition from 0.5 nM.

**4** In the venous thrombosis model, MA33H1 significantly reduced thrombus weights by 38 and 58.6% at 50 and 100 $\mu$g $kg^{-1}$ $min^{-1}$ i.v. respectively. This effect was inhibited by tranexamic acid. In the arterial model, MA33H1 significantly increased the delay to occlusive thrombus formation by 58 and 142% at 50 and 100 $\mu$g $kg^{-1}$ $min^{-1}$ i.v., and did not affect bleeding time at 300 $\mu$g $kg^{-1}$ $min^{-1}$ i.v.

**5** Thus, a monoclonal antibody which transforms PAI-1 to a t-PA substrate prevents thrombus formation in the rat with no effect on bleeding time at a higher dose.

**Keywords:** PAI-1 inhibition; plasminogen activators; rat venous thrombosis; arterial thrombosis; bleeding time

## Introduction

The formation of an intravascular thrombus depends upon the dynamic equilibrium between the endogenous haemostatic and fibrinolytic processes. The fibrinolytic pathway is regulated by endogenous inhibitors. In the case of tissue type plasminogen activator (t-PA), its action is controlled by the serpin plasminogen activator inhibitor type 1 (PAI-1). PAI-1 specifically and rapidly inactivates t-PA at concentrations in the physiological range (Kruithof *et al*., 1984). Like other serpins and their target enzymes, PAI-1 acts as a pseudosubstrate for t-PA; it forms a very stable complex with the active site of the enzyme thus rendering it catalytically inert. PAI-1 has been described as adopting various 'globular' conformations including an active form with the active centre in a reactive loop on the external surface, and an inactive or 'latent' form, where this centre is folded in a $\beta$ sheet inside the molecule (Mottonen *et al*., 1992).

The role of PAI-1 in the etiology of intravascular thrombus formation and the resistance to thrombolysis is increasingly recognized. Increased plasma levels of PAI-1 are correlated with several thrombotic diseases in man (Dawson & Henney, 1992). More recently, a 4G/5G polymorphism in the promoter region of the PAI-1 gene has been identified whereby homozygous 4G/4G subjects have higher plasma levels of PAI-1 (Dawson *et al*., 1993), and this genotype is significantly associated with a history of myocardial infarction (Eriksson *et al*., 1995; Ossei-Gerning *et al*., 1997) and coronary artery disease in sufferers from non-insulin-dependent diabetes mellitus (Mansfield *et al*., 1995).

Studies in animals have shown that manipulation of PAI-1 activity can have profound effects. Recombinant PAI-1 in its active form has been shown to prevent thrombolysis in a rabbit model of venous thrombosis where rt-PA alone caused almost 100% thrombolysis (Vaughan *et al*., 1992). Clot-bound PAI-1 inhibited endogenous fibrinolysis in a rat model of pulmonary emboli (Reilly *et al*., 1991; Hantgen, 1994). Furthermore, transgenic mice with a hyperexpression of human PAI-1 developed venous thrombosis in their hind limbs and tails immediately after birth. The pathological symptoms were maximal at 12 days and by $\approx$ 2 weeks the tail tips had sloughed off (Erickson *et al*., 1990). Inhibition of PAI-1 has been shown to promote thrombolysis. For example, Levi *et al*. (1992) showed that a monoclonal antibody against PAI-1 accelerated the lysis of human thrombi inserted in the rabbit jugular vein and inhibited thrombus extension both in the absence and in the presence of exogenous t-PA. The same group also showed that a PAI-1-neutralizing monoclonal antibody increased endogenous thrombolysis and prevented thrombus growth in a venous thrombosis model in the rabbit, and accelerated reperfusion and prevented reocclusion in a canine model of coronary artery thrombosis (Biemond *et al*., 1995). In addition, an $F_{ab}$-fragment of a PAI-1 inhibiting antibody has been shown to reduce thrombus growth and restore blood flow in a rat model of $FeCl_3$-induced carotid arterial thrombosis when administered after the thrombogenic stimulus (Van Giezen *et al*., 1997).

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In addition to active and latent forms of PAI-1 mentioned above, a further conformation has recently been described, which acts as a non-inhibitory substrate for t-PA (Declerck *et al*., 1992; Urano *et al*., 1992; Munch *et al*., 1993). Unlike the latent conformation which does not bind to the active site of t-PA, the substrate conformation reacts with t-PA resulting in cleavage of the $P_1-P_1'$ bond and regeneration of active t-PA (Declerck *et al*., 1992; Audenaert *et al*., 1994). A series of monoclonal antibodies has been prepared which are able to bind to active PAI-1 in such a way as to induce this substrate form of PAI-1, and it has been suggested that such a mechanism may confer antithrombotic/thrombolytic properties to such antibodies (Debrock & Declerck 1997). Moreover, these antibodies are able to cross-react with rabbit and porcine PAI-1 (Debrock & Declerck 1997). We therefore decided to examine the cross-reactivity of one of these antibodies, MA33H1, with rat PAI-1, and, if sufficient cross-reactivity was obtained, to evaluate the ability of MA33H1 to prevent thrombus formation in models of venous and arterial thrombosis in the rat, which we have previously shown to be sensitive to heparin and the thrombin inhibitor argatroban (Berry *et al*., 1994).

## Methods

### *Activity of the monoclonal antibody MA33H1 against human and rat PAI-1*

The monoclonal antibody MA33H1 used in the studies was purchased from Prof P Declerck, Katholieke Universiteit Leuven. MA33H1 (50, 100 and 200 ng $ml^{-1}$ final concentration) was incubated at 37°C for 15 min with 0.2 nM two-chain human t-PA (final concentration, Biopool, Umeå, Sweden) in the presence of recombinant human or rat PAI-1 (Molecular Innovations, Royal Oak, MI, U.S.A.) at a final concentration titrated to inhibit t-PA by 75% (usually 0.15 nM) in a 96-well microtitre plate. All the reagents were in buffer comprising 20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 80 (pH 7.8) in a total volume of 100 $\mu$l. At the end of the incubation period, residual t-PA activity was determined by adding 200 $\mu$l chromogenic substrate H-D-Ile-Pro-Arg-pNA (S2288, Chromogenix, Mölndal, Sweden) dissolved in 100 mM Tris-HCl, 0.1% Tween 80 (pH 8.4). The samples were then incubated for 2 h at 37°C in a Labsystems iEMS 96-well microtitre plate reader coupled to a Compaq 486 PC, during which time the optical density of the samples at 405 nm was measured every 5 min. Each plate contained a series of control wells (t-PA alone, t-PA + PAI-1, t-PA + PAI-1/2, and solvent blanks), and all reaction conditions were performed in triplicate wells. For each well, the $V_{max}$ of the reaction was determined using Biolyse and Biograph software (Labsystems), and PAI-1 inhibition was calculated for each antibody concentration with the above software. In a separate study, the effects of vitronectin and fibrin on the inhibitory activity of MA33H1 against human PAI-1 were studied. The antibody was incubated with PAI-1 in the presence of increasing concentrations of vitronectin (Molecular Innovations, Royal Oak, U.S.A.; 0.005 – 5 nM) or fibrin (Chromogenix, Mölndal, Sweden; 1 – 1000 ng $ml^{-1}$) as above, and the inhibition of t-PA activity was determined as above using the residual t-PA assay. The results obtained were expressed as the means ± s.e.mean per cent inhibition from three separate microtitre plates, where each plate was prepared using freshly prepared reagents.

### *Venous thrombosis in the rat*

Non-fasted male CD rats (340 – 370 g Charles River, France) were anaesthetized with sodium pentobarbitone (Sanofi, 60 mg $kg^{-1}$ i.p., 0.1 ml 100 $g^{-1}$ body weight). Anaesthesia was maintained by repeated administration of sodium pentobarbitone when appropriate. The left femoral and jugular veins were cannulated for the intravenous (i.v.) injection of drugs and thromboplastin respectively. The abdominal vena cava was exposed for the placing of vessel clamps. Thrombus formation was induced by the injection of 20 $\mu$g $kg^{-1}$ rabbit brain thromboplastin (La Technique Biologique, Paris, France) followed 10 s later by blood stasis in a 1 cm segment of the abdominal vena cava. Stasis was maintained for 15 min, after which time, the thrombus was removed and immediately weighed. MA33H1 or vehicle (Phosphate-buffered saline) were administered as a continuous i.v. infusions (0.07 ml $min^{-1}$) starting 30 min before the thromboplastin injection and continuing throughout the experiment (i.e. 45 min). For each treatment group the mean thrombus weight ± s.e.mean was determined, and tests for statistical significance between the treatment and control groups were performed by one way analysis of variance followed by Dunnett's Test using RS1 software. The percentage inhibition of thrombus formation was determined for each treatment group.

### *Arterial thrombosis in the rat*

Non-fasted male CD rats (430 – 500 g, Charles River, France) were anaesthetized as above. The right femoral vein was cannulated for i.v. drug administration. A segment (approximately 2 cm long) of the left carotid artery was exposed and dissected free of the vagus and surrounding tissue and fitted on the distal end with an appropriately sized Doppler flow probe (Triton Technology Inc. San Diego, CA, U.S.A.). A bipolar Diastre electrode was placed 1 cm proximal to the probe. Blood flow was measured with a Triton pulsed Doppler Flowmeter (system 6, model 200) and recorded on a Graphtec DMS 1000 chart recorder. A mechanical zero flow was determined by momentarily clamping the artery between the probe and the electrode. Thrombus formation was induced by the application of an electrical current of (3mA DC) for 5 min (with the flowmeter turned off during current application) to the external arterial surface by means of a DC stimulator (Service Electronique, Synthélabo Recherche, France). Blood flow was recorded for 30 min post-stimulation. When the flow declined to the predetermined zero flow (mechanical zero), the time in min to thrombus formation was noted. MA33H1 or vehicle were administered as above except that the infusions were started 30 min before thrombus induction (i.e. 65 min total drug administration). For each treatment group the mean time to occlusion ± s.e.mean was determined, and tests for statistical significance between the treatment and control groups were performed as above. The percentage increase in the time to occlusive thrombus formation was determined for each treatment group. If the vessels were still patent at the end of the observation period, a value of 30 min was ascribed for the sake of statistical analysis.

### *Rat tail-transection bleeding time*

Non-fasted male CD rats (320 – 340 g, Charles River, France) were anaesthetized as above. The right jugular vein was cannulated for i.v. drug administration. Bleeding was induced by section of the extremity of the tail 3 mm from the tip; the

tails were gently blotted with tissue paper every 2 min, and the time in min to cessation of bleeding was noted. Care was taken that no pressure was exerted on the tail tips, which could affect haemostasis. MA33H1 (300 $\mu$g kg$^{-1}$ min$^{-1}$) or its vehicle were administered as continuous i.v. infusions starting 30 min before tail transection and maintained throughout the experiment. For each treatment group, the mean bleeding time $\pm$ s.e.mean was determined, and tests for statistical significance between the treatment and control groups was performed using Student's *t*-test for unpaired samples.

## Results

### *Activity of the antibody MA33H1 against human and rat PAI-1*

The monoclonal antibody MA33H1 was shown concentration-dependently to inhibit recombinant human PAI-1 as from 10 ng ml$^{-1}$ with 44.1 $\pm$ 0.1% inhibition at 100 ng ml$^{-1}$ (Figure 1). MA33H1 had no inhibitory effect on recombinant rat PAI-1 at 10 ng ml$^{-1}$, but, at 50 and 100 ng ml$^{-1}$, the antibody was as effective at inhibiting rat PAI-1 as it was against human PAI-1 with 49.7 $\pm$ 1.0% inhibition at the latter concentration. This high degree of cross-reactivity of the antibody enabled its evaluation in models of thrombosis.

As shown in Table 1 the inhibitory activity of MA33H1 against human and rat PAI-1 was not altered in the presence of fibrin at concentrations up to 1 $\mu$g ml$^{-1}$. Vitronectin up to a concentration of 1 nM had no effect on the activity of MA33H1 against human PAI-1 (Table 2) although the antibody was inhibited by 44% in the presence of 5 nM vitronectin (N.B. the concentration of PAI-1 in the assay system was 0.15 nM). On the other hand, the inhibitory activity of the antibody against rat PAI-1 was concentration-dependently reduced by vitronectin, although a 33 fold molar excess of vitronectin was required to abolish the inhibition.

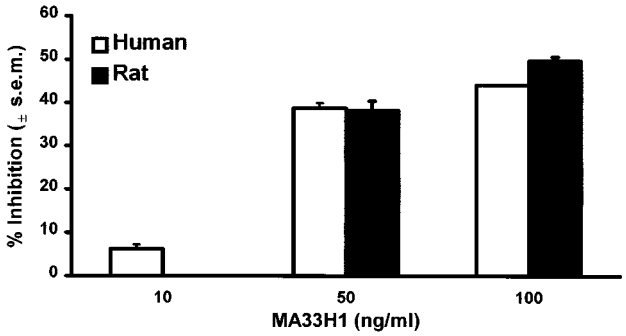


**Figure 1** Inhibition of human (open bars) and rat (filled bars) recombinant PAI-1 by MA33H1. The antibody was incubated with PAI-1 and t-PA before addition of a chromogenic substrate for t-PA. Each bar shows the mean % PAI-1 inhibition $\pm$ s.e. mean. ($n$ = three separate experiments).

**Table 1** Effects of fibrin on the inhibitory activity of 100 ng ml$^{-1}$ MA33H1 against human and rat PAI-1

| *Fibrin* (ng ml$^{-1}$) | *% inhibition* *Human rPAI-1* | *Rat rPAI-1* |
|---|---|---|
| 0 | 42.1 $\pm$ 2.5 | 42.5 $\pm$ 2.7 |
| 1 | 42.5 $\pm$ 4.1 | 44.8 $\pm$ 4.5 |
| 5 | 38.4 $\pm$ 2.4 | 40.8 $\pm$ 4.2 |
| 10 | 43.5 $\pm$ 3.2 | 46.5 $\pm$ 3.3 |
| 50 | 42.9 $\pm$ 3.1 | 44.5 $\pm$ 1.6 |
| 100 | 43.3 $\pm$ 2.7 | 45.3 $\pm$ 2.5 |
| 500 | 41.4 $\pm$ 1.5 | 45.0 $\pm$ 1.2 |
| 1000 | 40.3 $\pm$ 0.6 | 44.5 $\pm$ 2.5 |

MA33H1 was incubated with PAI-1 and t-PA in the presence of increasing concentrations of fibrin for 15 min at 37°C before addition of a chromogenic substrate. Results are expressed as mean $\pm$ s.e.mean per cent inhibition of PAI-1 activity ($n$ = 3 separate experiments).

### *Antithrombotic activity of MA33H1 in rat venous and arterial thrombosis*

The injection of thromboplastin followed by 15 min stasis in the abdominal vena cava leads to the formation of an erythrocyte-rich thrombus with wet weights ranging from 26.5 to 43.4 mg. Figure 2 shows that an intravenous infusion of MA33H1 leads to an apparent dose-dependent decrease in the thrombus weight with 38 and 59% decreases in thrombus weight at 50 and 100 $\mu$g kg$^{-1}$ min$^{-1}$ MA33H1 respectively. In a separate study, the effects of the coadministration of tranexamic acid (4 mg kg$^{-1}$ min$^{-1}$, Sigma, France) on the antithrombotic activity of 50 $\mu$g kg$^{-1}$ min$^{-1}$ MA33H1 were evaluated. Under these conditions, the reduction in thrombus wet weight observed with MA33H1 was abolished in the presence of tranexamic acid (Figure 3).

**Table 2** Effects of vitronectin on the inhibitory activity of 100 ng ml$^{-1}$ MA33H1 against human and rat PAI-1

| *Vitronectin* (nM) | *% inhibition* *Human rPAI-1* | *Rat rPAI-1* |
|---|---|---|
| 0 | 38.2 $\pm$ 3.6 | 38.5 $\pm$ 1.0 |
| 0.005 | 40.7 $\pm$ 2.6 | 41.9 $\pm$ 1.8 |
| 0.01 | 40.3 $\pm$ 3.3 | 42.5 $\pm$ 1.5 |
| 0.05 | 41.8 $\pm$ 3.1 | 41.1 $\pm$ 4.3 |
| 0.1 | 41.8 $\pm$ 1.4 | 34.9 $\pm$ 2.6 |
| 0.5 | 38.0 $\pm$ 3.6 | 25.6 $\pm$ 5.7 |
| 1.0 | 33.5 $\pm$ 3.2 | 14.3 $\pm$ 4.3 |
| 5.0 | 21.4 $\pm$ 3.4 | 1.2 $\pm$ 1.2 |

MA33H1 was incubated with PAI-1 and t-PA in the presence of increasing concentrations of vitronectin for 15 min at 37°C before addition of a chromogenic substrate. Results are expressed as means $\pm$ s.e.mean per cent inhibition of PAI-1 activity ($n$ = 3 separate experiments).

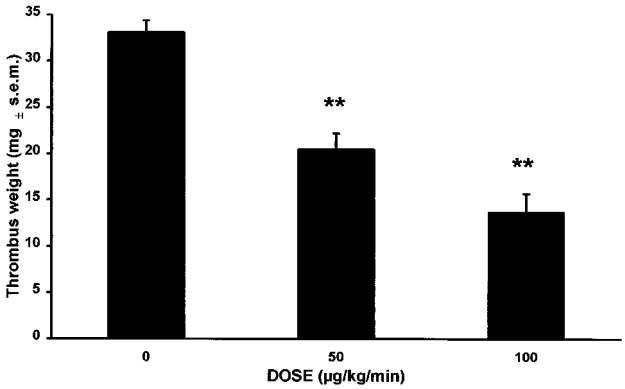


**Figure 2** Bar graph showing the effects of intra-venous infusions of MA33H1 on thrombus formation in the rat venous thrombosis model. Each bar shows the mean $\pm$ s.e.mean thrombus wet weight for each treatment group ($n$ = 6, except controls where $n$ = 10). The infusions were started 30 min before the injection of 20 $\mu$g kg$^{-1}$ thromboplastin followed by stasis of blood in the abdominal vena cava. Statistical significance compared with control values is denoted by asterisks where $^{**}P < 0.01$ vs controls, Dunnett's test.

In the arterial thrombosis model, application of an electric current to the adventitial surface of the carotid artery leads to the formation of a stable occlusive thrombus denoted by zero flow, which occurred at $7.7 \pm 0.6$ min (mean $\pm$ s.e.mean., $n=15$) after the end of the period of electrical stimulation. When the animals had been treated with MA33H1 there were significant and dose-related increases in the delay to vessel occlusion in animals receiving both 50 and 100 $\mu$g kg$^{-1}$ min$^{-1}$. This antithrombotic activity is shown in Figure 4.

### *Effects of MA33H1 on the rat tail-transection bleeding time*

Transection of the caudal extremity of the tail gave a bleeding time of $13.6 \pm 0.7$ min (mean $\pm$ s.e.mean., $n=5$). The bleeding time was slightly increased by treatment with 300 $\mu$g kg$^{-1}$ min$^{-1}$ MA33H1 to $15.6 \pm 2.6$ min (mean $\pm$ s.e.mean., $n=5$). This difference was not significant by Student's *t*-test.

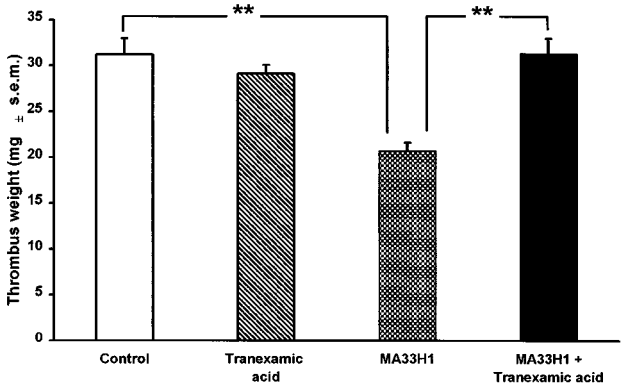


**Figure 3** Bar graph showing the effects of coadministration of tranexamic acid (4 mg kg$^{-1}$ min$^{-1}$) on the antithrombotic activity of MA33H1 (50 $\mu$g kg$^{-1}$ min$^{-1}$) in the venous thrombosis model. Each bar represents the mean $\pm$ s.e.mean thrombus weight for each treatment group (shown on the ordinate, $n=6$). Statistical significance for animals receiving MA33H1 alone (chequered bars) compared with controls and with animals receiving concomitant tranexamic acid treatment is denoted by asterisks where $^{**}P<0.01$ vs controls (□), and vs tranexamic acid and MA33H1 (■), Duncan's test.

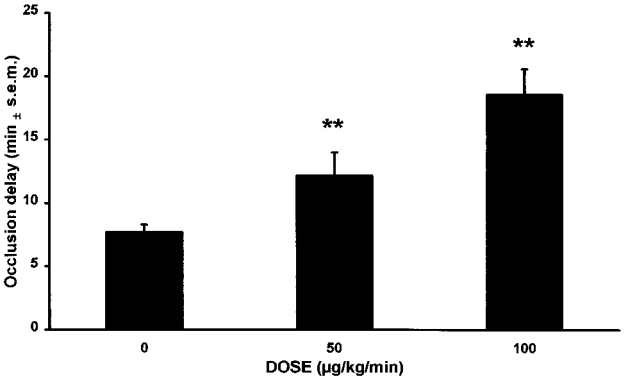


**Figure 4** Bar graph showing the effects of intra-venous infusions of MA33H1 on thrombus formation induced by a 3 mA d.c. electric current applied to the left carotid artery. Each bar shows the mean $\pm$ s.e.mean time in min to the formation of an occlusive thrombus as denoted by zero flow for each treatment group ($n=6$, except controls where $n=15$). The infusions were started 30 min before thrombus induction. Statistical significance compared with control values is denoted by asterisks where $^{**}P<0.01$ vs controls, Dunnett's test.

**Table 3** Relative prohaemorrhagic/antithrombotic therapeutic margins for MA33H1 vs heparin and the antithrombin argatroban in the rat

| | *MA33H1* | *Argatroban*[a] | *Heparin*[a] |
|---|---|---|---|
| M.D. arterial model (A) | 50 | 20 | 25 |
| $ED_{100}$ bleeding time (B) | >300 | 11 | 2.2 |
| B/A | >6 | 0.55 | 0.088 |

M.D. indicates minimal dose producing a statistically significant antithrombotic effect, $ED_{100}$ is the estimated dose producing a 100% increase in the bleeding time. Doses are expressed as $\mu$g kg$^{-1}$ min$^{-1}$. [a]Data for argatroban and heparin are taken from Berry *et al.* (1994).

## Discussion

The monoclonal antibody MA33H1 is one of a novel series of 'switching' antibodies which is capable of inducing a non-inhibitory substrate form of PAI-1. These antibodies recognize both active, latent PAI-1 and PAI-1 complexed with t-PA, and studies with a bacteriophage-display PAI-1 random epitope library show that they bind to PAI-1 between Glu 128 and Ala 156 of the PAI-1 molecule outside the reactive site loop (Debrock & Declerck, 1997; 1998). Moreover, as mentioned above, MA33H1 has been shown to cross react with rabbit and porcine PAI-1. In this study, we have shown that MA33H1 also reacts with recombinant rat PAI-1 as effectively as it does with recombinant human PAI-1 using a functional assay. Higher concentrations of the antibody were not studied here, since, firstly, our objectives were to ascertain that there was sufficient cross-reactivity between the two species for antithrombotic studies, and secondly, full concentration inhibition curves for MA33H1 have already been shown with human, murine and rabbit PAI by Debrock and Declerck (1997), where >80% inhibition was obtained with a molar ratio of antibody: antigen of 15. The binding site of MA33H1 partly comprises a region (Met110-Lys145 in human PAI-1) believed to be putative sites for the binding of vitronectin (Keijer *et al.*, 1991) and fibrin (Van Meijer *et al.*, 1994). Our studies with vitronectin, support the observation by Debrock and Declerck (1998) that MA33H1 binds to a region on human PAI-1 which is distinct from (albeit in close proximity to) the vitronectin binding site; however, in rat PAI-1 the two binding sites would appear to have some considerable degree of overlap. The fact that fibrin had no effect on PAI-1 inhibition by MA33H1 in either species clearly shows that the two binding sites are different.

Very few studies have addressed the possibility that PAI-1 inhibition leading to an increase in endogenous fibrinolytic capacity may prevent thrombus formation, although a series of synthetic diketopiperazine PAI-1 inhibitors have been shown to be antithrombotic in the electric current-induced carotid arterial thrombosis model in the rat (Charlton *et al.*, 1996; Barnes, *et al.*, 1996), and an $F_{ab}$ fragment of a PAI-1 neutralizing antibody attenuates thrombus formation in a $FeCl_3$-induced arterial thrombosis model (Van Giezen *et al.*, 1997). Our studies with MA33H1 confirm these observations that PAI-1 inhibition can prevent arterial thrombosis, and also show that PAI-1 inhibition is also able to prevent venous thrombosis in a rat model. Indeed, this is the first demonstration to our knowledge of the antithrombotic activity of a PAI-1 inhibitor in both arterial and venous thrombosis models in the same species. Moreover, MA33H1 exerted antithrombotic activity at the same doses in both venous and

arterial thrombosis, which distinguishes PAI-1 inhibition from anticoagulants where higher doses are required for antithrombotic efficacy in animal models of arterial thrombosis compared to models of venous thrombosis in the rat (Freund *et al*., 1990; Talbot *et al*., 1991; Berry *et al*., 1994). Furthermore, the antithrombotic effect of MA33H1 in the venous thrombosis model was abolished in the presence of tranexamic acid, which inhibits endogenous fibrinolysis by blocking the binding of plasminogen to fibrin *via* the high affinity lysine binding site (Hoylaerts *et al*., 1981). This implies that in MA33H1-treated animals that the thrombus was lysed as quickly as it was being formed and that PAI-1 is an important contributor to the stability of the intra-vascular thrombus.

MA33H1 has no effect on the rat tail transection bleeding time at 300 $\mu$g kg$^{-1}$ min$^{-1}$ i.e. at a dose which was six times greater than the lowest dose used to produce a statistically significant antithrombotic effect. We did not use higher doses, because of availability of the antibody. Although it would be unwise to use active doses in a crude tail-transection bleeding time model to predict an absolute haemorrhagic risk for a given compound, such information could be useful in providing information on the relative haemorrhagic risks of different therapeutic classes. Table 3 shows the activity of MA33H1 in the arterial thrombosis and tail-transection bleeding models in the rat compared with that of heparin and the synthetic thrombin inhibitor argatroban in the same models and using the same mode of administration, i.e. intravenous infusion so that the effects of differences in pharmacokinetics would be minimized (Berry *et al*., 1994). In these models, it would seem that MA33H1 and, by inference, PAI-1 inhibitors would have a better therapeutic margin than anticoagulant agents as antithrombotic agents in arterial thrombosis. Similarly, we have previously shown that the doses of heparin (but not argatroban) required to increase bleeding were in the same range as the active doses in the venous thrombosis model, which is in stark contrast to the data obtained with MA33H1.

To date, the *in vivo* studies on PAI-1 inhibition have focussed on antibodies or low molecular weight PAI-1 inhibitors which bind to or near the reactive centre loop of PAI-1. For example, competitive surface plasmon resonance studies suggest that the diketopiperazine XR5118 inhibits the interaction of PAI-1 with t-PA at the reactive centre loop, and XR5118 inhibits thrombus growth after thrombolysis in a rabbit model of thrombosis (Friederich *et al*., 1997). Our study shows that, in addition to direct inhibition of the binding of active PAI-1 to t-PA, the induction of a non-inhibitory substrate form of PAI-1 by antibodies such as MA33H1 can also lead to antithrombotic activity. Such a mechanism may offer a novel approach to antithrombotic therapy, since induction of the substrate form of PAI-1 would allow regeneration of the target protease, and would lead to an almost irreversible inhibition of the serpin, which would only be overcome by *de novo* protein synthesis. Since, for example, it has been shown that there is an increase in plasma PAI-1 antigen on cessation of rt-PA therapy (Rapold *et al*., 1991; Åstedt *et al*., 1993), in patients with unstable angina who had subsequent cardiovascular events (Wieczoric *et al*., 1994), and in patients who developed deep vein thrombosis after hip replacement (Eriksson *et al*., 1989), the normalization of PAI-1 levels by induction of its substrate form may offer an effective therapy with much reduced bleeding risks compared to currently available antithrombotic agents.

## References


ÅSTEDT, B., CASSLÉN, B., LECANDER, I., NILSSON, L. & NORGREN, L. (1993). Rebound increase of PAI-1 following local intra-arterial rt-PA infusion, a possible cause of reocclusion. *Blood Coagulation and Fibrinolysis,* **4,** 563 – 567.

AUDENAERT, A.-M., KNOCKAERT, I., COLLEN, D. & DECLERCK, P.J. (1994). Conversion of plasminogen activator inhibitor-1 from inhibitor to substrate by point mutations in the reactive centre loop. *J. Biol. Chem.,* **269,** 19559 – 19564.

BARNES, C.S., FAINT, R.W., DWYER, M.C., CRITCHLEY, D.J.P., TEMPLETON, D., FOLKES, A.J., CHARLTON, P.A. & BEVAN, P. (1996). Effects of XR5118 on PAI-1 and tPA activity and arterial thrombus formation in the anaesthetised rat. *Brit. J. Pharmacol.,* **118** (suppl.), 97P.

BERRY, C.N., GIRARD, D., LOCHOT, S. & LECOFFRE, C. (1994). Antithrombotic actions of argatroban in rat models of venous 'mixed' and arterial thrombosis, and its effects on the tail transection bleeding time. *Br. J. Pharmacol.,* **113,** 1209 – 1214.

BIEMOND, B.J., LEVI, M., CORONEL, R., JANSE, M.J., TEN CATE, A.W. & PANNEKOEK, H. (1995). Thrombolysis and reocclusion in experimental jugular vein and coronary artery thrombosis. Effects of a plasminogen-activator inhibitor type 1-neutralizing antibody. *Circulation,* **91,** 1175 – 1181.

CHARLTON, P.A., FAINT, R.W., BENT, F., BRYANS, J., CHICARELLI-ROBINSON, I., MACKIE, I., MACHIN, S. & BEVAN, P. (1996). Evaluation of a low molecular modulator of human plasminogen activator inhibitor-1 activity. *Thromb. Haemostas.,* **75,** 808 – 815.

DAWSON, S. & HENNEY, A. (1992). The status of PAI-1 as a risk factor for arterial and thrombotic disease: a review. *Atherosclerosis,* **95,** 115 – 117.

DAWSON, S.J., WIMAN, B., HAMSTEN, A., GREEN, F., HUMPHRIES, S. & HENNEY, A.M. (1993). The two allele sequences of a common polymorphism in the promoter of the plasminogen-activator inhibitor-1 (PAI-1) gene respond differently to interleukin 1 in HepG2 cells. *J. Biol. Chem.,* **268,** 10739 – 10745.

DEBROCK, S. & DECLERCK, P.J. (1997). Neutralization of plasminogen activator inhibitor-1 inhibitory properties: identification of two different mechanisms. *Biochim. Biophys. Acta.,* **1337,** 257 – 266.

DEBROCK, S. & DECLERCK, P.J. (1998). Identification of a functional epitope in plasminogen activator inhibitor-1, not localised in the reactive centre loop. *Thromb. Haemostas.,* **79,** 597 – 601.

DECLERCK, P.J., DE MOL, M., VAUGHAN, D.E. & COLLEN D. (1992). Identification of a conformationally distinct form of plasminogen activator inhibitor-1, acting as a non-inhibitory substrate for tissue-type plasminogen activator. *J. Biol. Chem.,* **267,** 11693 – 11696.

ERICKSON, L.A., FICI, G.J., LUND, J.E., BOYLE, T.P., POLITES, H.G. & MAROTTI, K.R. (1990). Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor 1 gene. *Nature,* **346,** 74 – 76.

ERIKSSON, B.I., ERIKSSON, E., GYZANDER, E., TEGER-NILSSON, A.-C. & RISBERG, B. (1989). Thrombosis after hip replacement relationship to the fibrinolytic system. *Acta Orthop. Scand.,* **60,** 159 – 163.

ERIKSSON, P., KALLIN, B., VANT'HOOFT, F.M., BAVENHOLM, P. & HAMSTEN, A. (1995). Allelic specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc. Natl. Acad. Sci.,* **92,** 1851 – 1855.

FREUND, M., CAZENAVE, J.-P., COURTNEY, M., DEGRYSE, E., ROITSCH, C., BERNAT, A., DELEBASSEE, D., DEFREYN, G. & MAFFRAND, J.-P. (1990). Inhibition by recombinant hirudins of experimental venous thrombosis and disseminated intravascular coagulation induced by tissue factor in rats. *Thromb. Haemostas.,* **63,** 187 – 192.

FRIEDERICH, P.W., LEVI, M., BIEMOND, B.J., CHARLTON, P., TEMPLETON, D., VAN ZONNEVELD, A.J., BEVAN P.J., PANNKOEK, H. & TEN CATE J.W. (1997). Novel low-molecular weight inhibitor of PAI-1 (XR5118) promotes endogenous fibrinolysis and reduces postthrombolysis thrombus growth in rabbits. *Circulation,* **96,** 916 – 921.

HANTGEN, R.R. (1994). PAI-1 released from cultured human endothelial cells delays fibrinolysis and is incorporated into the developing fibrin clot. *Fibrinolysis,* **8,** 104 – 112.

HOYLAERTS, M., LIJNEN, H.R. & COLLEN, D. (1981). Studies on the mechanism of the antifibrinolytic actions of tranexamic acid. *Biochim. Biophys. Acta.,* **673,** 75 – 85.

KEIJER, J., LINDERS, M., VAN ZONNENFELD, A.J., EHRLICH, H.J., DE BOER, J.P. & PANNEKOEK H. (1991). The interaction of plasminogen activator inhibitor-1 (PAI-1) with plasminogen activators (t-PA, u-PA) and fibrin: localization of interaction sites and physiological relevance. *Blood,* **78,** 401 – 409.

KRUITHOF, E.K.O., TRAN-THANG, C., RANSIJN, A. & BACHMANN, F. (1984). Demonstration of a fast acting inhibitor of plasminogen activators in human plasma. *Blood,* **64,** 907 – 913.

LEVI, M., BIEMOND, B.J., VAN ZONNEVELD, A.J., TEN CATE, A.W. & PANNEKOEK, H. (1992). Inhibition of plasminogen activator inhibitor-1 results in promotion of endogenous thrombolysis and inhibition of thrombus extension in models of experimental thrombosis. *Circulation,* **85,** 305 – 312.

MANSFIELD, M.W., STICKLAND, M.H. & GRANT P.J. (1995). Plasminogen activator inhibitor-1 (PAI-1) promoter polymorphism and coronary artery disease in non-insulin-dependent diabetes. *Thromb. Haemostas.,* **74,** 1032 – 1034.

MOTTONEN, J., STRAND, A., SYMERSKY, J., SWEET, R.M., DANLEY, D.E., GEOHEGAN, K.F., GERARD, R.D. & GOLDSMITH, E.J. (1992). Structural basis of latency of plasminogen activator inhibitor-1. *Nature,* **355,** 270 – 273.

MUNCH, M., HEEGAARD, C.W. & ANDREASEN, P.A. (1993). Interconversions between active, inert and substrate forms of denatured/refolded type-1 plasminogen activator inhibitor. *Biochim. Biophys. Acta.,* **1202,** 29 – 37.

OSSEI-GERNING, N., MANSFIELD, M.W., STICKLAND, M.H., WILSON, I.J. & GRANT, P.J. (1997). Plasminogen activator inhibitor-1 promoter 4G/5G genotype and plasma levels in relation to a history of myocardial infarction in patients characterised by coronary angiography. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 33 – 37.

RAPOLD, H.J., GRIMAUDO, V., DECLERCK, P.J., KRUITHOF, E.K.O. & BACHMANN, F. (1991). Plasma levels of plasminogen activator inhibitor type 1, $\beta$-thromboglobulin and fibrinopeptide A before during, and after treatment of acute myocardial infarction with Alteplase. *Blood,* **78,** 1490 – 1495.

REILLY, C.F., FUJITA, T., MAYER, E.J. & SIEGFRIED, M.A. (1991). Both circulating and clot-bound plasminogen activator inhibitor 1 inhibit endogenous fibrinolysis in the rat. *Arterioscler. Thrombos.,* **11,** 1276 – 1286.

TALBOT, M.D., AMBLER, J.A., BUTLER, K.D., LEES, C.M., MITCHELL, K.A., PETERS, R.F., TWEED, M.F. & WALLIS, R.B. (1991). The effects of recombinant desulphatohirudin on arterial thrombosis in rats. *Haemostasis,* **21** (suppl. 1), 73 – 79.

URANO, T., STRANDBERG, L., JOHANSSON, L.B. & NY, T. (1992). A substrate-like form of plasminogen activator inhibitor-1. Conversions between different forms by sodium dodecyl sulphate. *Eur. J. Biochem.,* **209,** 985 – 992.

VAN GIEZEN, J.J.J., WAHLUND, G., NERME, V. & ABRAHAMSSON, T. (1997). The $F_{ab}$-fragment of a PAI-1 inhibiting antibody reduces thrombus size and restores blood flow in a rat model of arterial thrombosis. *Thromb. Haemostas.,* **77,** 965 – 969.

VAN MEIJER, M., KLEIN GEBBINK, R., PREISSNER, K. & PANNEKOEK, H. (1994). Determination of the vitronectin binding site on plasminogen activator inhibitor type 1 (PAI-1). *FEBS Lett.,* **352,** 342 – 346.

VAUGHAN, D.E., DECLERCK, P.J., VAN HOUTTE, E., DE MOL, M. & COLLEN, D. (1992). Reactivated recombinant plasminogen activator inhibitor (rPAI-1) effectively prevents thrombolysis in vivo. *Thromb. Haemostas.,* **68,** 60 – 63.

WIECZORIC, I., LUDLAM, C.A. & FOX, K. (1994). Tissue type plasminogen activator and plasminogen activator inhibitor activities as predictors of adverse events in unstable angina. *Am. J. Cardiol.,* **74,** 424 – 429.

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