

Tiplaxtinin, a Novel, Orally Efficacious Inhibitor of Plasminogen Activator Inhibitor-1: Design, Synthesis, and Preclinical Characterization

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Abstract: Indole oxoacetic acid derivatives were prepared and evaluated for in vitro binding to and inactivation of human plasminogen activator inhibitor-1 (PAI-1). SAR based on biochemical, physiological, and pharmacokinetic attributes led to identification of tiplaxtinin as the optimal selective PAI-1 inhibitor. Tiplaxtinin exhibited in vivo oral efficacy in two different models of acute arterial thrombosis. The remarkable preclinical safety and metabolic stability profiles of tiplaxtinin led to advancing the compound to clinical trials.

Elevated levels of plasminogen activator inhibitor-1 (PAI-1) have been implicated in acute and chronic diseases, including deep vein thrombosis,¹ atherosclerosis,² and type 2 diabetes.³ Plasma PAI-1 is also elevated in postmenopausal women and has been proposed to contribute to the increased incidence of cardiovascular disease in this population.⁴ PAI-1 is the most important physiologic regulator of the plasminogen activation system through its inhibition of its target serine proteases, tissue plasminogen activator (tPA), and urokinase plasminogen activator (uPA).⁵ Significant elevations of PAI-1 lead to stabilization of arterial and venous thrombi, which contribute respectively to coronary arterial occlusion in postmyocardial infarction⁶ and venous thrombosis following postoperative recovery from orthopedic surgery.⁷ Studies in PAI-1 null mice suggest that PAI-1 inhibition is associated with improvement in pathophysiologic processes, including thrombosis,⁸ atherosclerosis,⁹ and pulmonary fibrosis,¹⁰ each of which is regulated through plasmin generation. The detrimental effects of PAI-1 in atherosclerosis may also be caused by a separate effect of PAI-1 in inhibiting smooth muscle cell migration leading to plaque rupture.^{11,12} Strategies for reducing PAI-1 have included the development of inhibitory antibodies that neutralize PAI-1, and studies have demonstrated that anti-PAI-1 antibodies can effectively neutralize PAI-1 activity in vitro and in vivo.^{13,14} The use of antibodies for the treatment of diseases associated with the chronic eleva-

tion of plasma PAI-1 is limited by their lack of oral activity, fostering the development of small-molecule inhibitors of PAI-1. Research in this area has been challenging, in part because of the conformational plasticity of PAI-1. For example, the metastable conformation of active PAI-1¹⁵ has prohibited crystal structure determination and hindered rational drug design approaches, requiring a unique approach to the development of small-molecule inhibitors. As with all active serine protease inhibitors (serpins), the conformational strain of the exposed reactive center loop results in a preferred relaxation by inserting the N-terminal portion of the loop as strand 4 of the major β -sheet A, thereby adopting an inactive, or "latent" conformation.¹⁶ While the active form of PAI-1 is structurally unstable with a plasma half-life of ~ 1 h, it is the conformation that is inhibitory toward tPA and uPA and therefore the target for small-molecule interaction. Recent research efforts have resulted in the identification of several compounds that are reported to inhibit PAI-1, including the salicylic acid derivative HP129,¹⁷ the anthranilic acid derivative AR-H029953XX,¹⁸ the diketopiperazine XR5118,^{19,20} and the butadiene derivative T-686²¹ (Figure 1), yet none of them have shown in vivo activity.

Our efforts to identify small-molecule PAI-1 inhibitors started with the high-throughput screening (HTS) of our compound libraries. Substructure searching using scaffolds of the confirmed hits produced multiple classes of compounds, which were clustered into series based on distinct chemical scaffolds. Common to all these series was the existence of a carboxylic acid or an acid bioisostere. This structural feature has also been common to PAI-1 inhibitors reported in the literature (Figure 1). Of the series we identified, the indole oxoacetic acid scaffold proved to be the most successful of the structures explored and ultimately culminated in the discovery of tiplaxtinin. Here, we describe our efforts leading to the discovery and advancement of tiplaxtinin to clinical trials.

The initial HTS hit in the indole oxoacetic acid series was **1**. Testing of related indole analogues identified various chemical leads with general structure **2** (Figure 2). Improved activity was realized with **2** in which R¹ is an aryl group directly attached to the indole, R² is a bulky group such as a benzyl group, and R³ is a hydrogen atom or a small alkyl group. In a subset of compounds (**3**), where the acidic moiety was represented by an oxoacetic acid and R¹ is a phenyl ring, SAR optimization focused on exploring the optimal position for the phenyl linkage on the indole and the effect of substituents on the phenyl and benzyl groups.

The 6-phenyl-1-benzyl analogue (**3.1**) showed moderate activity with an IC₅₀ of 26 μ M (Table 1). While a trifluoromethoxy group in the 4-position of the phenyl ring enhanced activity, substitution on the 4-position of the benzyl group (4-F or 4-*t*-Bu) diminished activity (**3.3** and **3.4**). However, upon translocation of the substituted phenyl group from the 6-position of the indole ring to the 5-position, a set of compounds having a *t*-Bu substituent in the 4-position of the benzyl ring (**3.5**, **3.6**, **3.7**, **3.8**) exhibited good inhibitory activity.

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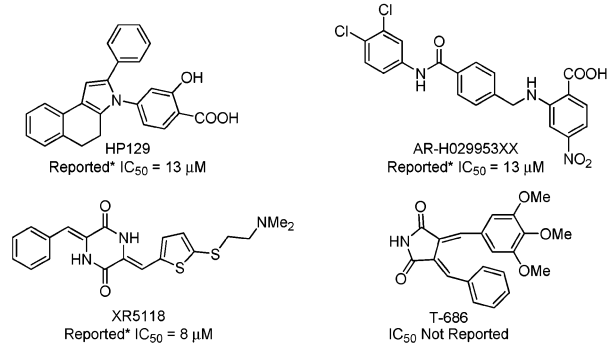


Figure 1. Structures of PAI-1 inhibitors. * For comparative IC_{50} values, see ref 17.

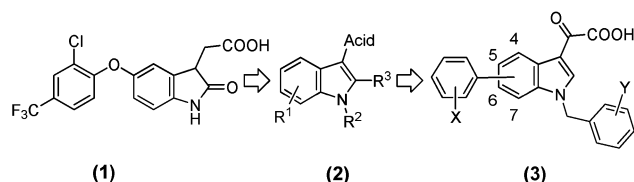


Figure 2. Lead development.

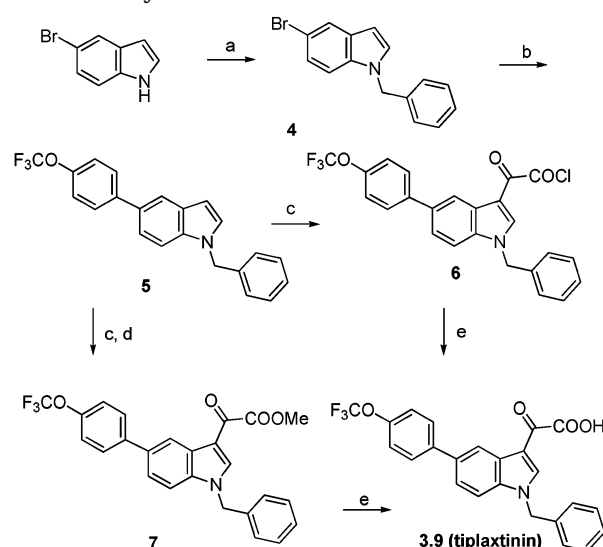
Table 1. Optimization of Compounds of Series 3

	position	X	Y	IC_{50} (μM)
3.1	6	H	H	26
3.2	6	4-OCF ₃	H	17
3.3	6	4-OCF ₃	4- <i>t</i> -Bu	>50
3.4	6	H	4-F	35
3.5	5	4-OCF ₃	4- <i>t</i> -Bu	16
3.6	5	3-OMe	4- <i>t</i> -Bu	21
3.7	5	3-Me	4- <i>t</i> -Bu	8.6
3.8	5	3- <i>t</i> -Bu	4- <i>t</i> -Bu	10
3.9	5	4-OCF ₃	H	2.7
3.10	5	3-OCF ₃	H	9.8
3.11	4	4-OCF ₃	H	>50
3.12	7	4-OCF ₃	H	10

Ultimately, the unsubstituted benzyl derivative (**3.9**) was a more potent compound with an IC_{50} of 2.7 μM . Subsequently, translocation of the 4-trifluoromethoxyphenyl group of **3.9** to the 7-position yielded a compound with moderate activity (**3.12**, IC_{50} = 10 μM). The corresponding 4-regioisomer (**3.11**) was found to be a weaker inhibitor (IC_{50} > 50 μM).

3.9 was prepared in four steps from starting materials that are commercially available (Scheme 1). The synthesis was straightforward and amenable to large-scale preparation. No chromatographic separations were required at any step of the synthesis. Purification of the final product was achieved through crystallization. Preparation of 1-benzyl-5-bromoindole (**4**) was initially carried out by alkylation of 5-bromoindole with benzyl bromide in THF using sodium hydride as a base. Because of the inherent hazards associated with sodium hydride on a large scale, it was replaced with potassium *tert*-butoxide without affecting yield or purity during the scale-up campaign. Palladium-catalyzed coupling of the bromoindole **4** with 4-trifluoromethoxyphenylboronic acid under Suzuki reaction conditions afforded **5**. Reaction of indole derivative **5** with oxalyl chloride in tetrahydrofuran yielded the oxo acid chloride derivative **6**, which was found to be stable and crystalline and was therefore used for controlling purity at the penultimate stage of the synthesis. Alternatively, quenching of **6** with methanol produced the methyl ester **7**, which was

Scheme 1. Synthesis of **3.9**^a



^a (a) Benzyl bromide, *t*-BuOK, DMF; (b) 4-CF₃O-Ph-B(OH)₂, Pd(OAc)₂, Bu₄NBr, K₂CO₃, dioxane, water; (c) oxalyl chloride, THF; (d) MeOH; (e) KOH, acetone, water, HCl.

also found to be highly crystalline. Hydrolysis of the acid chloride **6** or the ester **7** under basic conditions followed by acidification and crystallization furnished compound **3.9** as a high-purity substance with the desired crystal form corresponding to a polymorph²² at 165 °C. Multi-kilogram batches of **3.9** were produced using this synthetic route (sequences a, b, c, e) with an overall yield of greater than 65%.

The *in vitro* characterization of PAI-1 inhibition by **3.9** employed three separate and distinctly different assays. In the primary screen for PAI-1 inhibition, preincubation of recombinant human PAI-1 with tPA resulted in the complete inhibition of the enzymatic action of tPA with a synthetic substrate as determined spectrophotometrically. Addition of **3.9** to human PAI-1 restored the proteolytic activity of tPA, indicating successful inhibition of PAI-1. A more sensitive secondary assay quantified residual active PAI-1 by antibody binding following incubation with various concentrations of the compound and was used to determine the IC_{50} . In this assay, uninhibited active human PAI-1 is captured on a tPA-coated culture plate and the non-SDS dissociable complex is quantified using a polyclonal antibody. In the final assay, binding of **3.9** to PAI-1 using fluorescence quenching was used to determine direct binding and affinity of the compound for PAI-1. Binding assays employed PAI-1 mutants (S119C or S149C) labeled with the fluorescent tag nitrobenzo-2-oxa-1,3-diazole (NBD). Binding isotherms were generated and dissociation constants (K_d) were determined. Inhibition assays functionally linked the observed PAI-1 binding event to the extent of inhibition of the serine protease.

3.9 was evaluated for efficacy in two separate animal models of acute thrombosis. In a rat model of thrombosis, an occlusive thrombus was chemically induced in the carotid artery of instrumented, anesthetized rats. Drug substance was administered orally to the animals prior to the induction of the thrombus. Experiments were designed where the thrombus formation coincided with the T_{max} of the drug in the test group. Blood flow,

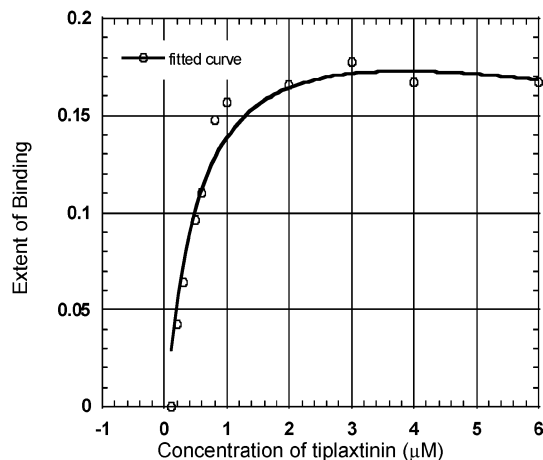


Figure 3. Binding isotherm of tiplaxtinin (**3.9**).

Table 2. Oral Efficacy of Tiplaxtinin (**3.9**) in a Rat Thrombosis Model

treatment ^a	occlusion time (min)	arterial occlusion (%)	carotid flow reduction (%)	thrombus wt (mg)
vehicle control	11.5 ± 0.4	100	100.0 ± 0.0	1.6 ± 0.03
tiplaxtinin (1 mg/kg)	50.5 ± 9.5 ^b	25	47.3 ± 11.3 ^b	0.5 ± 0.15 ^b

^aData are mean values ± SEM for four rats per group.

^bSignificantly different from value of the vehicle control group at $p < 0.05$.

time to occlusion, thrombus weights, and arterial patency were assessed in drug-treated animals and compared to those of vehicle controls. In a second model, an electrolytic injury was induced in the lumen of the coronary artery of the anesthetized dog, which also results in occlusive thrombus formation, but the injury differs from the rodent model because of its anatomic site and specific endothelial location of damage. In the canine model, spontaneous reperfusion of the coronary artery in the presence of **3.9** was monitored over a 4 h period following injury.

Results of *in vitro* experiments indicated that **3.9** inhibited PAI-1 with an IC_{50} of 2.7 μM as determined by the antibody method. By use of fluorescent spectroscopy, **3.9** bound to the NBD-labeled S119C PAI-1 mutant selectively with a K_d of 480 nM (Figure 3). This binding event was saturable and was associated with inhibition of the protein. Furthermore, the binding affinity toward latent PAI-1 was greatly reduced with an apparent K_d of 5 μM .

In the rat carotid thrombosis model, oral administration of **3.9** at 1 mg/kg increased time to occlusion and prevented the carotid blood flow reduction when compared to the vehicle group. As shown in Table 2, all of the vehicle control rats exhibited thrombosis with an average time to occlusion of 11 min and a complete reduction of carotid flow of 100%. Conversely, those rats receiving **3.9** at 1 mg/kg *po* exhibited an average time to occlusion of over 50 min and a carotid blood flow reduction of approximately 50%. **3.9** treatment was also associated with reduction in thrombus weight and increased arterial patency. These improvements in hemostatic endpoints occurred without effects on heart rate, blood pressure, or bleeding.

3.9 was also efficacious in the second model of acute thrombosis in which injury is induced directly to the

Table 3. Oral Efficacy of Tiplaxtinin (**3.9**) in a Dog Thrombosis Model

treatment ^a	occlusion time (min)	arterial occlusion	coronary flow reduction (%)	spontaneous reperfusion
vehicle control	24.7 ± 5.5	3/3 (100%)	100.0 ± 0.0	0/3 (0%)
tiplaxtinin (3 mg/kg)	71.6 ± 9.8 ^b	3/3 (100%)	66.0 ± 22.1	3/3 (100%)

^aData are mean values ± SEM for three dogs per group.

^bSignificantly different from the value of the vehicle control group at $p < 0.05$.

endothelium of the canine coronary artery. In this model, the arterial insult resulted in a 100% incidence of coronary occlusion, determined as zero measurable blood flow, in vehicle and drug-treated animals (Table 3). Importantly, however, spontaneous coronary reperfusion indicative of active fibrinolysis was observed only in animals receiving **3.9** orally. Arterial flow and time to occlusion were also improved in the drug-treatment group, but again, no bleeding was observed.

Pharmacokinetic parameters of **3.9** were evaluated in the same two species used for determination of efficacy, male SD rats, and male beagle dogs. In rats, following a single *iv* bolus of 1 mg/kg (vehicle, 20%DMSO/80%PEG₂₀₀), **3.9** exhibited an apparent half-life of 2.95 ± 1.15 h, a mean maximum plasma concentration (C_{max}) of 4.95 ± 1.53 $\mu g/mL$, and an $AUC_{0-\infty}$ of 2.45 ± 0.42 $\mu g \cdot h/mL$. Repeated oral dose administration of **3.9** to rats at the 1 mg/kg efficacious dose resulted in a C_{max} of 0.23 $\mu g/mL$, a half-life of 4.1 h, and an AUC_{0-24} ($\mu g \cdot h/mL$) of 0.96, which with a MW of 439 is approximately equal to the *in vitro* IC_{50} . In addition, plasma concentrations of **3.9** increased with increasing doses in a linear manner following oral administration, and the relative bioavailability was 93%. Similarly, in dogs following a single *iv* bolus of 1 mg/kg, the apparent half-life was 3.73 ± 0.40 h, C_{max} was 13.44 ± 3.53 $\mu g/mL$, and the $AUC_{0-\infty}$ was 20.54 ± 5.04 $\mu g \cdot h/mL$. Following oral administration of **3.9** to dogs at the efficacious dose of 3 mg/kg, the C_{max} was 2.14 $\mu g/mL$, the half-life was 5.42 h, and the relative bioavailability was 32%.

On the basis of lack of significant toxicity, the no toxic effect level (NTEL) in rats was set as equal to the allowed maximum dose of 2000 (mg/kg)/day. Exposure at this dose ($AUC \approx 1508 \mu g \cdot h/mL$) was 1500-fold the efficacious exposure ($AUC \approx 1 \mu g \cdot h/mL$). The NTEL in dogs was 100 (mg/kg)/day, which was equivalent to a safety multiple of 600-fold the efficacious exposure ($AUC \approx 613 \mu g \cdot h/mL$ at NTEL vs $AUC \approx 1 \mu g \cdot h/mL$ at efficacy).

3.9 was nongenotoxic in the Ames assay, the *in vitro* human peripheral blood lymphocyte (HPBL) chromosomal aberration assay, and the *in vivo* micronucleus test. There were no effects on cardiovascular, central nervous, or respiratory systems in rats and dogs.

3.9 was shown to be selective for PAI-1 as indicated by selectivity assays against a number of different proteins, including tPA and α_1 -antitrypsin, the serpin most closely related to PAI-1. The compound was also inactive in 40 separate assays in the NovaScreen (Hanover, MD) peripheral side effect profile, which included neurotransmitter, endocrine, and enzymatic targets.

In conclusion, tiplaxtinin (**3.9**) was identified as a potent and selective PAI-1 inhibitor using a variety of in vitro assays, and in vivo efficacy was demonstrated in multiple models of acute arterial thrombosis. Oral efficacy was observed in an in vivo rat thrombosis model at plasma concentrations near the IC₅₀. In the canine coronary artery thrombosis model, treatment with tiplaxtinin was associated with spontaneous reperfusion of the occluded coronary vessel indicative of active fibrinolysis. Tiplaxtinin has exceptional oral bioavailability, is metabolically stable, exhibits large safety multiples in animal toxicology studies, and is easily synthesized in bulk quantity. These chemical and physiological characteristics of tiplaxtinin, together with its unique profibrinolytic activity without associated bleeding, make it an excellent candidate for clinical development.

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Supporting Information Available: Experimental details, spectroscopic data, results from elemental analyses for **3.9**, methods for direct PAI-1 in vitro activity assays, and in vivo thrombosis models. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- 3.9** exhibited polymorphism. Different crystal forms have different dissolution rates, which affect drug absorption leading to different levels of drug exposure following oral administration. The crystal form of **3.9** with 165 °C melting point was found to be stable and reproducible.

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