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Low Molecular Weight Antagonists of Plasminogen Activator Inhibitor-1: Therapeutic Potential in Cardiovascular Disease

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Abstract

Plasminogen activator inhibitor-1 (PAI-1; SERPINE1) is the major physiologic regulator of the plasmin-based pericellular proteolytic cascade, a modulator of vascular smooth muscle cell (VSMC) migration and a causative factor in cardiovascular disease and restenosis, particularly in the context of increased vessel transforming growth factor- β 1 (TGF- β 1) levels. PAI-1 limits conversion of plasminogen to plasmin (and, thereby, fibrin degradation) by inhibiting its protease targets urokinase and tissue-type plasminogen activators (uPA, tPA). PAI-1 also has signaling functions and binds to the low density lipoprotein receptor-related protein 1 (LRP1) to regulate LRP1-dependent cell motility that, in turn, contributes to neointima formation. PAI-1/uPA/uPA receptor/LRP1/integrin complexes are endocytosed with subsequent uPAR/LRP1/integrin redistribution to the leading edge, initiating an “adhesion-detachment-readhesion” cycle to promote cell migration. PAI-1 also interacts with LRP1 in a uPA/uPAR-independent manner triggering Jak/Stat1 pathway activation to stimulate cell motility. PAI-1 itself is a substrate for extracellular proteases and exists in a “cleaved” form which, while unable to interact with uPA and tPA, retains LRP1-binding and migratory activity. These findings suggest that there are multiple mechanisms through which inhibition of PAI-1 may promote cardiovascular health. Several studies have focused on the design, synthesis and preclinical assessment of PAI-1 antagonists including monoclonal antibodies, peptides and low molecular weight (LMW) antagonists. This review discusses the translational impact of LMW PAI-1 antagonists on cardiovascular disease addressing PAI-1-initiated signaling, PAI-1 structure, the design and characteristics of PAI-1-targeting drugs, results of *in vitro* and *in vivo* studies, and their clinical implications.

Introduction

PAI-1 is a member of the serine protease inhibitor (SERPIN) superfamily, consisting of over 40 proteins with approximately 35% homology and representing more than 10% of the total plasma protein [1–7]. PAI-1 is a single-chain, glycosylated polypeptide, similar in tertiary structure to most other SERPINs and is comprised of three β -sheets (A, B, C), α -helices (A–I) and a reactive center loop (RCL) situated in the carboxy terminus [7]. PAI-1 inhibits serine protease activity by forming a reversible Michaelis-like 1:1 stoichiometric complex with the target proteinase, followed by creation of a non-covalent acyl intermediate, and ultimately the formation of covalent ester bond between the carboxyl group the active SERPIN and the hydroxyl group of the serine protease, mimicking the normal substrate to proteinase interaction [8,9]. Upon formation of the covalent inhibitory complex, the peptide

bond in the reactive center loop that joins Arg³⁴⁶ and Met³⁴⁷ (P1-P1'), mimics the normal substrate of the proteinase and is cleaved rendering PAI-1 inactive, an event that has aptly led to the designation of PAI-1 as a "suicide inhibitor" [10,11].

PAI-1 Structure and Conformations

PAI-1 exists in three structurally and functionally distinct conformations, active, latent, and cleaved (substrate) [12–14]. The active conformation interacts with the target proteinase and is unstable (half-life approximately 2 hours at 37°C, pH 7.4) and converts spontaneously into the latent form, which cannot bind its protease targets [13,15,16]. PAI-1 is cleaved by proteinases at the P1-P1' site in the reactive center loop, a reaction that can occur in the absence of a covalent complex [17–19]. In the latent form, the N-terminus of the reactive center loop inserts into β -sheet A forming a new β -strand (s4A) producing an unusual loop structure and conformational change in the reactive center disrupting the peptide bond between Arg³⁴⁶ and Met³⁴⁷ (P1-P1') [20]. Alternatively, PAI-1 can be cleaved by target proteases at the peptide bond between Arg³⁴⁶ and Met³⁴⁷ (P1-P1'), resulting in the insertion of the N-terminal end of the reactive center loop into β -sheet A. The C-terminus of the reactive site loop forms strands 1C in β -sheet C producing a 70Å separation of the P1 and P1' residues [19,21–23]. Clarification of these PAI-1 conformational crystal structures has driven the effort to design low molecular weight PAI-1 antagonists.

PAI-1 Function and Role in Pathological States

PAI-1 can be found in platelets and in the plasma. Tissue injury results in an approximate 10-fold increase in plasma PAI-1 upon platelet activation [24–26]. PAI-1 rapidly inhibits tPA and uPA (tissue type and urokinase-like plasminogen activators, respectively) with second order rate constants approximately $3.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ [16,27,28]. The primary role of the plasminogen activator system is to generate the active enzyme plasmin from its precursor, plasminogen, a key step in the fibrinolytic cascade. Indeed, PAI-1 deficiency in humans results in a hyperfibrinolytic state and abnormal bleeding after trauma or surgery [29–33]. Moreover, the generation of plasmin leads to the activation of matrix metalloproteinases and extracellular matrix (ECM) degradation [34–36]. PAI-1 (SERPINE1) is the major physiologic regulator of the plasmin-based pericellular proteolytic cascade, a modulator of vascular smooth muscle cell (VSMC) migration and a causative factor in cardiovascular disease and restenosis (Figure 1), particularly in the setting of increased TGF- β 1 [37]. Inhibition of the fibrinolytic system by PAI-1 over expression in transgenic mice results in the spontaneous development of thrombi and pervascular fibrosis [38,39]. PAI-1^{-/-} mice exhibit significantly reduced VSMC density in the neointima (compared to wild type counterparts) in response to oxidative stress-induced vessel injury and in the balloon-catheterized carotid artery as well as in atherosclerosis prone ApoE^{-/-} mice [37,40,41]. Importantly, clinical findings are in agreement with these data as, after transluminal coronary angioplasty, PAI-1 activity was significantly greater in patients with restenosis compared to those without clinical recurrence [42]. This has significant translational ramifications. Atherosclerosis and arterial restenosis are associated with a dysregulation of physiological reconstruction. While it is well-accepted that medial smooth muscle cell migration and proliferation, followed by ECM synthesis are critical in the development of vascular lesions, there remains the need for a better understanding of molecular mechanisms that contribute to smooth muscle cell migration and neointimal development [43–46].

Beyond regulating fibrinolysis, PAI-1 functions in a signaling capacity to impact low density lipoprotein receptor-related protein 1 (LRP1)-dependent cell migration. PAI-1 forms a complex that consists of PAI-1/uPA/uPAR (uPA receptor)/LRP1/integrin which, in turn, is

endocytosed resulting in uPAR/LRP1/integrin redistribution to the leading edge leading front of the cell, causing an “adhesion-deadhesion-readhesion” cycle that promotes cell migration. Redistribution of integrins is not required for deadhesion, but for migration upon redistribution at the leading edge, while uPAR and uPA are not required for migration, but instead deadhesion [47–52]. Furthermore, recycling of uPAR to the leading edge allows for induction of matrix degradation, which supports the motile phenotype [53]. PAI-1 can also bind to LRP1 in a uPA/uPAR-independent manner and activates Jak/Stat1 signaling inducing cell migration through Stat1-mediated transcriptional regulation of genes required for cell motility [47,49,54]. Importantly, all three forms of PAI-1 (active, cleaved, latent) are able to interact with LRP1 and increase LRP1-mediated cell migration through activation of Jak/Stat1 signaling at low concentrations [47].

Clearly, the implications of plasminogen activation go beyond just fibrinolytic control and extend to processes including cell migration and adhesion. This cascade has been functionally implicated in various disease states including thrombosis, atherosclerosis, restenosis, fibrosis, and cancer and has driven the effort to design small molecular weight antagonists of PAI-1. Early studies on PAI-1 inhibition focused on (a) utilizing monoclonal antibodies that convert PAI-1 to the latent or cleaved form and (b) peptides that correspond to the reactive site of PAI-1. While initial investigations provided important data regarding the utilization of PAI-1 inhibition in various disease states and facilitated the mapping sites of LMW antagonist binding, their poor oral bioavailability precluded clinical adaptability. Therefore, there is growing interest in investigating the efficacy of specific, potent, low molecular weight compounds on the inhibition of PAI-1.

Low Molecular Weight Antagonists

Several low molecular weight antagonists of PAI-1 have been discovered and characterized (Table 1). The first were diketopiperazines (XR330 and XR334) and, from that template, more potent antagonists were designed (XR1853, XR5082, XR5967, XR1121) which inhibit PAI-1 by inducing the transition from active PAI-1 to non-reactive PAI-1 [55–58]. By inhibiting the interaction of tPA/uPA and PAI-1 in a rat carotid artery thrombus model, XR334, XR5082 and XR1853 effectively increased fibrinolysis *in vivo* [55].

Other antagonists that act similarly include AR-H2029953XX and fendosal. AR-H2029953XX is an anthranalic acid derivative of the known fibrinolysis antagonist flufenamic acid [44–47]. The acidic function of AR-H2029953X is required for binding to PAI-1 in the vicinity of Arg76, Arg115 and Arg118, which are above and behind helix E and between β sheet A and helix D, a region implicated in the binding of LRP [2,47,59].

The negatively charged LMW antagonists ANS, bis-ANS and 1-dodecyl sulphuric acid and the positively charged XR-5118 overlap and localize around hD and hE and β -sheet A in the flexible joint region of PAI-1, which becomes accessible for the reactive center loop to insert during the reaction with proteinases [58,60,61]. This conformational rearrangement causes the reactive site to become inaccessible, thereby preventing tPA and uPA from binding potentially resulting in the inhibition of LRP binding due to the location of the binding site [61]. Of these compounds only XR-5118 has shown *in vivo* efficacy by increasing tPA activity and reduced rat arterial thrombus growth [62].

Compounds that insert into the s4A position of β -sheet A as a mock molecule that prevents the activity of PAI-1 have also been designed. Tiplaxtinin and TM5007, indole oxoacetic acid derivatives insert in the s4A position and effectively inhibit PAI-1 activity [63,64]. Furthermore, these compounds inhibit the PAI-1/tPA complex formation and importantly, both Tiplaxtinin and TM5007 are metabolically stable, non-toxic and showed good oral bioavailability and *in vivo* efficacy in a rat thrombosis model [63]. Finally, high

concentrations of non-ionic amphiphilic compounds, such as Triton X-100 cause an inhibition of PAI-1 and a butadiene derivative, T-686 was found to inhibit PAI-1 synthesis and augment PAI-1 activity induced by vascular injury and reduced atherosclerotic lesions in rabbits [65–67].

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Abbreviations

PAI-1	Plasminogen activator inhibitor-1
SERPINE1	Serine protease inhibitor clade E member 1
RCL	Reactive center loop
LMW	Low molecular weight
uPA	Urokinase plasminogen activator
tPA	Tissue-type plasminogen activator
LRP1	Low density lipoprotein receptor-related protein 1

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Clinical Implications

PAI-1 is the major physiological regulator of the plasmin proteolysis system and a critical component of the cell migratory machinery. High levels of PAI-1 have been implicated in several pathophysiological disease states, including fibro proliferative diseases of the cardiovascular system. Significant effort has gone into the design, synthesis and characterization of PAI-1 antagonists, however, there are no clinically applicable inhibitors available to date. As PAI-1 regulates cardiovascular disease largely through fibrinolysis and LRP1-dependent cell migration, it is important to determine the mechanism through which PAI-1 inhibitors abrogate cardiovascular pathogenesis. This Editorial describes several low molecular weight inhibitors with clinical potential including the diketopiperazine derivatives (XR330, XR334, XR1853, XR5082, XR5967, and XR1121), indole oxoacetic acid derivatives (TM5007 and Tiplaxtinin) and several others (AR-H2029953XX, fendosal, ANS, bis-ANS and 1-dodecyl sulphuric acid). Of these only XR334, XR5082, XR1853, XR5118, Tiplaxtinin and TM5007 have shown *in vivo* efficacy and only XR5118 has the potential to inhibit LRP1-dependent migration. Cell motility is a major contributor of pathological diseases including, but not limited to, cancer, fibrosis, restenosis and atherosclerosis. In this regard, it would appear prudent to continue to assess the translational impact of LMW PAI-1 inhibitors that specifically interfere with LRP1 binding.

Figure 1. PAI-1 is expressed in response to injury to the carotid artery

PAI-1 expressions at the protein (A) and mRNA transcript (B) levels were assessed in two different models of carotid injury. Placement of a carotid “cuff” in *APOE*^{-/-} mice maintained on a high fat diet results in development of a neointima in which PAI-1 is evident in the stromal compartment as well as in smooth muscle cells that constitute the luminal plaque (A; **immunohistochemistry**). Rats were subjected to balloon-catheter endothelial denudation injury of the left carotid artery. One week later, RNA was extracted from the injured carotids as well as the contralateral control arteries. Northern blotting using ³²P-labeled cDNA probes to PAI-1 and A50 indicated that PAI-1 was expressed in the injured vessel but not in the control carotid artery (B; **northern analysis**).

Table 1

Low molecular weight PAI-1 antagonists.

Mechanism of Action	Designation	IC ₅₀	Assay	Reference
Induces reactive center loop inaccessibility	1-dodecyl sulfuric acid	15.1 ± 3.4 μM	Direct uPA assay	[60,68]
	ANS	116 μM	Direct uPA assay	[57,60,68,69]
	Bis-ANS	0.57 μM	Direct uPA assay	[60,68,69]
	Tiplaxtinin	40 ± 7 μM	tPA-mediated plasmin generation assay	[63]
	TM5007	29.2 ± 4.2 μM	tPA-mediated plasmin generation assay	[63]
	XR5118	15 ± 1 μM	Plasmin generation assay	[62]
Inactivating	AR-H2029953XX	54 ± 18 μM	Plasmin generation assay	[57,60,69]
	Fendosal	36 ± 1 μM	Plasmin generation assay	[57]
	XR11211	0.2 ± 0.015 μM	Plasmin generation assay	[56]
	XR1853	62 ± 10 μM	Plasmin generation assay	[55,57]
	XR330	30 μM	tPA-mediated plasmin generation assay	[58]
	XR334	51 ± 7 μM	tPA-mediated plasmin generation assay	[55,58]
	XR5082	14.5 ± 0.5 μM	tPA-mediated plasmin generation assay	[55,57]
	XR5967	800 nM	Direct uPA assay	[70]
Inhibits PAI-1 synthesis	T-686	Not reported	N/A	[67]
Non-ionic detergent	Triton X-100	Not reported	N/A	[65,66]