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Neutralization of Plasminogen Activator Inhibitor I (PAI-1) by the Synthetic Antagonist, PAI-749, Via a Dual Mechanism of Action

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Running Title Page:

Running title: Inactivation of PAI-1 by a small molecule PAI-1 antagonist

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Nonstandard abbreviations:

PAI-1, plasminogen activator inhibitor type 1; PAI-2, plasminogen activator inhibitor type 2, PAI-749, [1-Benzyl-3-pentyl-2[6-(1*H*-tetrazol-5-ylmethoxy)naphthalene-2-yl]-1*H*-indole; PA, plasminogen activator; tPA, single chain tissue type plasminogen activator; 2c-tPA, two-chain tissue type plasminogen activator; uPA, urokinase type plasminogen activator; NBD, N,N'-dimethyl-N-(acetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PAI-NBD119, PAI-1 tagged with NBD at amino acid position 119; Fl, fluorescence; PAB, *p*-aminobenzamidine; HNEP, 0.1 M Hepes, 0.1 M NaCl, pH 7.4, 1 mM EDTA, 0.1% PEG8000; HNEPA, HNEP + 2 mM apo-10; DMSO, dimethylsulfoxide; Apo-10, dimethyldecylphosphine oxide; BN-PAGE, blue native polyacrylamide gel electrophoresis, VN, vitronectin

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ABSTRACT

PAI-749 is a potent and selective synthetic antagonist of plasminogen activator inhibitor 1 (PAI-1) that preserved tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activities in the presence of PAI-1 (IC_{50} values: 157 and 87 nM, respectively). The fluorescence (Fl) of fluorophore-tagged PAI-1 (PAI-NBD119) was quenched by PAI-749; the apparent K_d (254 nM) was similar to the IC_{50} (140 nM) for PAI-NBD119 inactivation. PAI-749 analogs displayed the same potency rank order for neutralizing PAI-1 activity and perturbing PAI-NBD119 Fl; hence, binding of PAI-749 to PAI-1 and inactivation of PAI-1 activity are tightly linked. Exposure of PAI-1 to PAI-749 for 5 min (sufficient for full inactivation) followed by PAI-749 sequestration with Tween-80 micelles yielded active PAI-1; thus, PAI-749 did not irreversibly inactivate PAI-1, a known metastable protein. Treatment of PAI-1 with a PAI-749 homolog (producing less assay interference) blocked the ability of PAI-1 to displace *p*-aminobenzamidine from the uPA active site. Consistent with this observation, PAI-749 abolished formation of the SDS-stable tPA/PAI-1 complex. PAI-749-mediated neutralization of PAI-1 was associated with induction of PAI-1 polymerization as assessed by native gel electrophoresis. PAI-749 did not turn PAI-1 into a substrate for tPA; however, PAI-749 promoted plasmin-mediated degradation of PAI-1. In conclusion, PAI-1 inactivation by PAI-749 using purified components can result from a dual mechanism of action. Firstly, PAI-749 binds directly to PAI-1, blocks PAI-1 from accessing the active site of tPA, and abrogates formation of the SDS-stable tPA/PAI-1 complex. Secondly, binding of PAI-749 to PAI-1 renders PAI-1 vulnerable to plasmin-mediated proteolytic degradation.

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Plasminogen activator inhibitor 1 (PAI-1) is a rapidly acting inhibitor of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Dellas & Loskutoff, 2005). PAI-1 is a member of the serpin class of serine protease inhibitors that characteristically produce SDS-stable complexes with their cognate protease targets (Silverman et al, 2001). Formation of the acyl-enzyme adduct between PAI-1 and the protease involves initial formation of a Michaelis-type noncovalent complex without significant conformational change, followed by reversible acylation, and irreversible reactive loop conformational changes that trap the protease in a covalent complex (Olson et al, 2001). Two other conformation states of PAI-1 are known. Firstly, the acyl-enzyme adduct between PAI-1 and tPA (or uPA) can be hydrolyzed to form cleaved (inactive) PAI-1 and regenerate active plasminogen activator (PA) (Declerck et al, 1992). Secondly, active PAI-1 can undergo a spontaneous large conformation change that gives rise to an inactive (latent) state of the inhibitor (Levin & Santell, 1987; Mottonen et al, 1992).

PAI-1 plays a pivotal role in a myriad of physiological processes that involve activation of plasminogen (Dellas & Loskutoff, 2005). High levels of PAI-1 activity are associated with a broad spectrum of pathophysiological states including thrombosis, cancer, inflammation, neurodegenerative and possibly metabolic diseases (Dellas & Loskutoff, 2005). Thus, neutralization of PAI-1 has been championed as a promising strategy to intervene in a number of diverse disease states involving suppressed extracellular proteolysis.

Small molecule synthetic antagonists of PAI-1 have been described (Charlton et al, 1996; Friederich et al, 1997; Neve et al, 1999; Gils et al, 2002; Elokdah et al, 2004; Liang et al, 2005; Einholm et al, 2003; Gorlatova et al, 2007). The metastable conformation of PAI-1 along with the intricacies of the pathway by which PAI-1 inhibits tPA affords numerous potential opportunities whereby a PAI-1 inhibitor can block PAI-1 activity. In this report, we probe the mechanism of action of a novel synthetic PAI-1 antagonist, PAI-749 (Fig. 1 inset; Elokdah et al, manuscript in preparation) using purified components. Our investigation reveals that PAI-749

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can neutralize PAI-1 activity via a dual mechanism of action: (a) direct inhibitory impact on PAI-1 activity and (b) rendering PAI-1 to be vulnerable to plasmin-mediated degradation.

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MATERIALS AND METHODS

Materials - PAI-749**,** 1-Benzyl-3-pentyl-2-[6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1H-indole; Compound B**,** 1-Benzyl-2-[5-methyl-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; Compound C**,** 1-Benzyl-2-[5-chloro-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; Compound D, 1-Benzyl-2-[5-bromo-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; Compound E, 2-[5-Chloro-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1-methyl-3-pentyl-1H-indole; Compound F, 1-Methyl-2-[5-methyl-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; Compound G, 2-[5-Bromo-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1-[2-(trifluoromethoxy)benzyl]-1H-indole; Compound H, 1-(4-tert-Butylbenzyl)-3-pentyl-2-[6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1H-indole were synthesized as described elsewhere (Elokdah et al, manuscript in preparation). Compounds were prepared as 1 mM stocks in dimethylsulfoxide (DMSO) and diluted into aqueous buffer (maintaining a final 1% DMSO concentration in all assays). Human PAI-1 (both active and latent), human PAI-1 variant (Ser119 to Cys) tagged with N,N'-dimethyl-N-(acetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD), Lys-plasmin, Glu-plasminogen, two-chain tPA (2c-tPA), high molecular weight uPA, human α_2-antiplasmin, monomeric vitronectin and human antithrombin III were from Molecular Innovations (Southfield, MI). Human recombinant single-chain tPA (tPA) (Activase^{TM}) was produced by Genentech (South San Francisco, CA). Spectrozyme tPA, Spectrozyme uPA, Spectrozyme FXa and plasminogen activator inhibitor type 2 (PAI-2) were from American Diagnostica (Greenwich, CT). HEPES free acid, aprotinin, *p*-aminobenzamidine (PAB), D-Val-Leu-Lys-pNA (plasmin substrate) and SAR-Pro-Arg-pNA (trypsin substrate) were from Sigma-Aldrich (St. Louis, MO). PEG-8000 was from USB (Cleveland, OH). Dimethyldecyl-phosphine oxide (apo-10) was from Calbiochem (San Diego, CA). Human α_1-antitrypsin and human pancreatic trypsin were from Athens Research and

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Technology (Athens, GA). Human factor Xa was from Enzyme Research Laboratories (South Bend, IN). Fondaparinux sodium (Arixtra™) was manufactured by GlaxoSmithKline (London, UK). Novex pre-cast gels (4-12% Bis-Tris gels), SilverXpress Staining kit, 4 X NuPAGE LDS sample prep buffer, Native PAGE Novex Bis-Tris gel system (4-16% Bis Tris gels) and SEE BLUE Plus 2 molecular weight standard were from Invitrogen (Carlsbad, CA). For molar concentration determinations, the following relative molecular weights were used: M_r of 43,000 for PAI-1; M_r of 66,000 for tPA; M_r of 83,000 for plasmin.

Assay of functional PAI-1 activity - PAI-1 (12 or 24 nM) was mixed with varying amounts of PAI-749 (or DMSO control) for 5 min at room temperature (final volume = 480 µl) in 0.1 M Hepes, 0.1 M NaCl, pH 7.4, 1 mM EDTA, 0.1% PEG-8000, 2 mM apo-10 (HNEPA buffer). The nonionic detergent, apo-10, was included in the assay buffer to stabilize PA activity. Apo-10 at 2 mM (which is below the critical micelle concentration of 4.6 mM) does not interfere with PAI-1 activity or the ability of PAI-749 to neutralize PAI-1 (in contrast to nonionic detergents such as Tween-80 and Triton X-100; data not shown). Twenty µl aliquot of tPA, 2c-tPA or uPA (10 or 20 nM final concentration) was added, and the samples were placed at room temperature for 10 min. Seventy five µl of each sample (performed in triplicate) was transferred to individual wells of a 96 well microtiter dish containing 25 µl Spectrozyme tPA (500 µM final) or Spectrozyme uPA (250 µM final). Reactions were continuously monitored at A_{405} in kinetic mode using a SPECTRAmax 340PC plate spectrophotometer (Molecular Devices). The IC_{50} values for PAI-749 were obtained using nonlinear regression (sigmoidal) curve fit analysis (GraphPad Prism; GraftPad Software Inc.).

Serpin selectivity assays - Selectivity assays for other serpins were performed essentially according to the PAI-1 and sc-tPA assay protocol (described above) with the following assay

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specific conditions. PAI-2 selectivity assay: PAI-2, 25 nM; 2c-tPA, 10 nM; Spectrozyme tPA, 500 μM. α_2-antiplasmin selectivity assay: α_2-antiplasmin, 7.5 nM; plasmin, 5 nM; D-Val-Leu-Lys-pNA, 200 μM. α_1-antitrypsin selectivity assay: α_1-antitrypsin, 50 nM; trypsin, 2.5 nM; SAR-Pro-Arg-pNA, 50 μM; HNEPA buffer was spiked with 10 mM $CaCl_2$. Antithrombin III selectivity assay: antithrombin III, 10 nM; LMW heparin (fondaparinux), 10 nM, factor Xa, 1 nM; Spectrozyme FXa, 100 μM.

SDS-PAGE analysis of PAI-1 and tPA reaction products - Samples (generated as described above) were mixed with 1/3 volume of 4 X NuPAGE LDS sample buffer without reducing agents, heated to 70^{o}C for 10 min, and fractionated by SDS-PAGE using NuPAGE (4-12%) Novex Bis-Tris gels. Proteins were visualized with the SilverXpress Staining Kit according to the manufacturer's instructions. Gels were analyzed with a flatbed scanner at 600 dpi and the resultant images were analyzed using Quantity One software (Bio-Rad, Hercules, CA). All bands were below software pixel intensity saturation plateaus. Local background correction for each band was calculated based on the average intensity of pixels immediately surrounding the defined area of analysis. Global background correction for each band was calculated based on the average pixel intensity of a defined section across the entire gel judged to free of protein staining. No significant difference in EC_{50} was apparent using either method of background correction.

Blue native PAGE (BN-PAGE) analysis of PAI-1 - PAI-1 (20 nM) was mixed with DMSO control (1% final) or PAI-749 for 60 min at room temperature. Samples (60 μl) were mixed with 1/3 volume of 4 X native gel sample buffer or 4 X LDS sample buffer. The former were subjected to BN-PAGE performed essentially as described (Schägger et al, 1991) using commercially available reagents. The latter were fractionated by SDS-PAGE as described above. Proteins were visualized by silver staining.

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Binding of compounds to PAI-1 using NBD-labeled PAI-1 variants - Fluorescence (Fl) measurements with NBD-labeled PAI-1 variants were performed using a LS50B fluorimeter (Perkin Elmer, Shelton CT). Excitation was at 480 nm; emission was at 500 to 600 nm (scan mode) or 525 nm (kinetic mode). Slit widths were 10 nm for both the excitation and emission settings. Experiments were performed in HNEPA buffer at room temperature. Experiments depicted in Figs. 2A, 3 and 4 involved exposure of PAI-NBD119 or latent PAI-NBD119 (50 nM) to DMSO vehicle (1% final) or various concentrations of PAI-749 (or PAI-749 analogs) for 5 min prior to assessment of Fl spectra. The latent PAI-NBD119 was prepared by incubation of active PAI-NBD119 at 37^{o}C in the dark for 16 hr. "Kinetic mode" experiments were also performed to probe the inhibitory mechanism of PAI-749. Fig. 2C: PAI-NBD119 (50 nM) was mixed with vehicle, PAI-749 (1 μM) or tPA (100 nM) and assayed immediately (over a 5 min interval). Parallel samples involved mixing PAI-NBD119 (50 nM) with PAI-749 (1 μM) or tPA (100 nM) for 5 min at room temperature, followed by addition of tPA (100 nM) or PAI-749 (1 μM), respectively, and the samples were assayed immediately (over a 5 min interval).

PAB displacement experiments to assess occupancy of the PA active site by PAI-1 - PAI-1 (1 μM) or PAI-1 pretreated (for 2 min) with 2 μM Compound B (closely-related analog of PAI-749 with lesser Fl signal interference; see Table 1 for its identity) was mixed with 0.5 μM uPA (or 2c-tPA) that was pretreated with 200 μM PAB. After 5 min, samples (600 μl final volume) were scanned using the fluorometer (excitation = 325 nm; emission scan from 340-400 nm). The emission signal at 355 nm was used to calculate active site occupancy of uPA (or 2c-tPA) by PAB.

Assaying for residual inhibition of PAI-1 after transient exposure to PAI-749 - Human PAI-1 (20 nM) was mixed with PAI-749 (1μM) or DMSO in HNEP buffer. The final DMSO concentration

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in all samples was 1%. Samples were prepared in duplicate. After 5 or 60 min at room temperature, 0.1% Tween-80 was added to one set of samples (which sequesters PAI-749 in ensuing detergent micelles). tPA (20 nM) was added to the appropriate samples. After 10 min, 75 µl of each sample was mixed with 25 µl Spectrozyme tPA (500 µM) and residual tPA activity was monitored at A_{405nm} for 10 min using a SPECTRAmax 340PC plate spectrophotometer.

Assaying for the impact of vitronectin on the ability of PAI-749 to inactivate PAI-1 - Human PAI-1 (24 nM) was mixed with PAI-749 (2 µM), VN (250 nM) or BSA (250 nM) in HNEPA buffer. The final DMSO concentration in all samples was 1%. Samples were prepared in duplicate. After 5 min at room temperature, VN, BSA or PAI-749 was added to certain samples as shown in Table 4. After 5 min, tPA (20 nM) was added to all samples. After 10 min, 75 µl of each sample was mixed with 25 µl Spectrozyme tPA (500 µM) and residual tPA activity was monitored at A_{405nm} for 10 min using a SPECTRAmax 340PC plate spectrophotometer.

Neutralization of PAI-1 by plasmin - Mixtures of active or latent PAI-1 (100 nM) and 1 µM PAI-749 (or DMSO control) were equilibrated for 5 min at room temperature. Reactions were initiated by the addition of plasmin (10 nM) (with and without aprotinin), incubated at 25°C for 30 min, quenched with 4 x LDS sample prep buffer and heated at 70°C for 10 min. Samples were fractionated by SDS-PAGE (4-12% Bis-Tris NOVEX pre-cast gels) and visualized by silver staining as described above.

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RESULTS

PAI-749 preserved tPA and uPA activity in the presence of PAI-1 - PAI-749 (Fig. 1, inset) dose-dependently blocked PAI-1 mediated inactivation of tPA activity towards its low molecular weight amidolytic substrate, Spectrozyme tPA (Fig. 1). The IC_{50} value of PAI-749 for preservation of tPA activity was 157 +/- 9 nM. Likewise, PAI-749 dose-dependently prevented PAI-1 mediated inactivation of uPA activity towards its low molecular weight substrate, Spectrozyme uPA (Fig. 1). The IC_{50} value of PAI-749 for blocking PAI-1 mediated inhibition of uPA was 87 +/- 3 nM. The ability of PAI-749 to preserve tPA and uPA activities towards Glu-plasminogen in the presence of PAI-1 was also demonstrated (data not shown).

PAI-749 displays selectivity for PAI-1 as compared to other serpin class inhibitors - The selectivity of PAI-749 for PAI-1 was evaluated against a panel of other serpins and their target proteases. Pretreatment of plasminogen activator inhibitor type 2 (PAI-2) (25 nM) with vehicle control or PAI-749 (5 μM) and subsequent addition to tPA (10 nM) yielded 62% and 48% inhibition of tPA activity, respectively. Pretreatment of α_2-antiplasmin (7.5 nM) with vehicle or PAI-749 (5 μM) and subsequent addition to plasmin (5 nM) caused 88% and 58% inhibition of plasmin activity, respectively. Pretreatment of antithrombin III (10 nM) with vehicle or PAI-749 (5 μM) and subsequent addition to factor Xa (1 nM) in the presence of fondaparinux (10 nM) produced 72% and 46% inhibition of Factor Xa activity, respectively. Lastly, pretreatment of α_1-antitrypsin (50 nM) with vehicle or PAI-749 (5 μM) and subsequent addition to trypsin (2.5 nM) caused 54% and 52% inhibition of trypsin activity, respectively. Hence, only slight neutralization of antithrombin III, α_2-antiplasmin, and PAI-2 was observed in the presence of 5 μM PAI-749 while α_1-antitrypsin was fully refractory to the effects of PAI-749 (at 5 μM). Control experiments established that PAI-749 (5 μM) did not directly impact the activity of the proteases

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used for these selectivity assays. Potential solubility problems at higher concentrations of PAI-749 in assay buffer precluded a more rigorous evaluation of the potency of the compound towards these other serpins. Nevertheless, these data clearly show that PAI-749 displayed marked selectivity for PAI-1 relative to other serpins.

PAI-749 is a direct, rapid, and reversible antagonist of PAI-1 – Treatment of a PAI-1 variant tagged with the NBD fluorophore at position 119 (PAI-NBD119) with PAI-749 for 5 min caused concentration-dependent quenching of the Fl signal (Fig. 2A). An apparent K_d value of 254 nM was deduced from the Fl signal perturbation at 525 nm (Fig. 2B). This value is less than 2-fold different than the IC_{50} (142 nM) of the PAI-NBD119 variant for tPA (data not shown). The change in the Fl spectrum was no different when the treatment interval was extended to 1 hr (data not shown). Moreover, similar results were obtained with a different Fl-tagged PAI-1 species (PAI-NBDP1′) in which the NBD moiety was present at the P1′ position of the reactive loop region of PAI-1 (data not shown).

PAI-NBD119 was used to assess the rapidity of PAI-749 binding to PAI-1 (Fig. 2C). The Fl signal of PAI-NBD119 was stable over the course of the 5 min observation period (progress curve 1). Addition of PAI-749 to PAI-NBD119 produced a rapid decline in the Fl signal (progress curve 2). The bulk of the Fl change occurred within the brief time interval (~15 sec) between reagent addition, sample mixing and cuvette placement. Progress curve #2 best fit to a single exponential curve fit. The limiting Fl quench signal was asymptotically reached by ~5 min.

We aimed to firmly establish that PAI-749 binding deduced from the compound-triggered Fl change of PAI-NBD119 was linked to inactivation of PAI-1. While the close similarity between the IC_{50} and apparent K_d values of PAI-749 for PAI-NBD119 is consistent with this notion, we evaluated the IC_{50} and apparent K_d values of several closely-related compounds in the PAI-749 chemical series to more fully assess if these 2 estimates for binding

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affinity reflect the same binding event (Table 1). All compounds differed from PAI-749 at the R_1/R_2 positions depicted in the structural template. Remarkably, the same rank order of potency was observed for this set of compounds with respect to both the IC_{50} and apparent K_d determinations (Fig. 3). The apparent K_d values are consistently higher than the corresponding IC_{50} values of all tested compounds for reasons inherent to the different assay methodologies that are yet to be defined. Nevertheless, these data establish that the binding events that perturb the Fl signal and neutralize PAI-1 activity are tightly linked.

A novel experimental strategy was used to test if inhibition of PAI-1 by PAI-749 was sustained after limited exposure to the compound. This approach exploited the fortuitous finding that PAI-749 was sequestered by Tween-80 detergent micelles (data not shown). PAI-1 was treated with PAI-749 for either 5 or 60 min; subsequently, the samples were exposed to buffer without or with 0.1% Tween-80 for the remainder of the experimental protocol. Tween-80 itself did not impact the ability of PAI-1 to inhibit tPA (Table 2: compare samples 1 & 2, 4 & 5, 7 & 8, 10 & 11). The approximate 2-fold greater tPA activity in the presence of Tween-80 reflected the ability of the nonionic detergent to stabilize tPA activity (perhaps by minimizing nonspecific absorption of tPA to the multiwell assay plate). In the absence of Tween-80, pretreatment of PAI-1 with PAI-749 (for 5 min) and subsequent addition of tPA (sample 3) is associated with marked preservation of tPA activity (i.e. indicative of PAI-1 neutralization). However, pretreatment of PAI-1 with PAI-749 for 5 min (in the absence of Tween-80), followed by exposure to Tween-80 and subsequent addition to tPA (sample 6) yielded virtually complete suppression of tPA activity (i.e. failure of PAI-749 to inhibit PAI-1). Hence, limited exposure of PAI-1 to PAI-749 is insufficient to elicit a sustained inhibitory effect. Interestingly, when PAI-1 was pretreated with PAI-749 for 60 min, the subsequent addition of Tween-80 was largely ineffective at reversing the inhibitory effect of PAI-749 (Table 2; compare samples 7 & 9, 10 & 12). Data shown below may provide the explanation why Tween-80 mediated reversibility is impacted by the exposure time between PAI-1 and PAI-749.

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Elucidating the mechanism of action of PAI-749 - The latent form of PAI-NBD119 was produced by incubation of active PAI-NBD119 at 37^{o}C for 16 hr. A time dependent change of the Fl signal and concomitant loss of PAI-1 inhibitory activity was observed (data not shown). Analysis of the samples by reverse fibrin zymography (which reactivates latent PAI-1) showed minimal loss of PAI-1 inhibitory activity (data not shown). These observations established that the latent form of PAI-NBD119 was generated.

The Fl signal displayed by latent PAI-NBD119 in the presence of PAI-749 was markedly different from that of active PAI-NBD119 treated with PAI-749 (Fig. 4: grey dashed scan vs. black dashed scan, respectively). Moreover, the Fl signal of latent PAI-NBD119 was only slightly perturbed by PAI-749 (compare grey solid and grey dashed scans), in contrast to that of active PAI-NBD119 (compare black solid and black dashed scans). These results showed that PAI-749 did not induce formation of the latent form of PAI-1 (a conclusion corroborated by additional findings described below). Moreover, the very slight perturbation of the Fl signal of latent PAI-1 in the presence of PAI-749 suggests that the affinity of PAI-749 for latent PAI-1 is low. However, we can not exclude the less likely possibility that PAI-749 bound to latent PAI-NBD119 but failed to perturb the Fl signal of the NBD-tag (unlike with active PAI-NBD119).

The canonical product of the reaction between PAI-1 and tPA is an SDS-stable complex. PAI-749 caused concentration dependent inhibition of the formation of the SDS-stable tPA/PAI-1 complex (Fig. 5A). The decrease in the abundance of the tPA/PAI-1 complex with increasing amounts of PAI-749 was accompanied by concomitant increases in both the tPA and (uncleaved) PAI-1 bands. Densitometry analysis revealed that the EC_{50} for blockade of the SDS-stable tPA/PAI-1 complex was 190 nM (Fig. 5B), a value that is very similar to the IC_{50} deduced from the activity assay described above. PAI-749 also interfered with formation of the SDS-stable complex between uPA and PAI-1; the concentration dependence of the PAI-749 effect matched the impact on preservation of uPA activity (data not shown).

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The effect of PAI-749 on the production of the cleaved form of PAI-1 was very slight if at all (Fig. 5A). A subtle biphasic concentration dependence of PAI-749 on the generation of the cleaved form of PAI-1 might be evident. At intermediate concentrations of PAI-749, there was a modestly enhanced formation of the cleaved species; however, with steadily increasing amounts of PAI-749, the generation of the cleaved species was abolished. In any event, PA-mediated cleavage of PAI-1 in the presence of PAI-749 does not appear to be a major contributor to neutralization of PAI-1 by PAI-749.

The marked ability of PAI-749 to block formation of the SDS-stable complex between PAI-1 and tPA could very likely reflect interference with an upstream event in the reaction pathway governing the multistep interaction between tPA and PAI-1 (Olson et al, 2001). To shed light on the actual step impacted by PAI-749, we examined the effect of a PAI-749 analog on the ability of PAI-1 to displace *p*-aminobenzamidine (PAB) from the PA active site. For this study we used Compound B (a closely-related analog of PAI-749; see Table 1 for its identify) instead of PAI-749 because the former posed lesser interference with the Fl signal of PAB (data not shown). In addition, uPA was used because it produced a more robust signal than tPA or 2c-tPA; however, similar conclusions were drawn from studies with 2c-tPA (data not shown). Binding of PAB to the uPA active site increased the intrinsic Fl signal of PAB (Table 3, sample 1; value depicts signal augmentation normalized to PAB control). As expected, addition of PAB to PAI-1 had no effect on the PAB Fl signal (sample 2). Treatment of the uPA/PAB complex with PAI-1 (sample 3) reduced the PAB Fl signal to yield a value that was indistinguishable from “no uPA” (sample 2). This result agreed with published data showing that PAI-1 displaced PAB from the active site of 2c-tPA (Olson et al, 2001). Compound B had little impact on the enhanced Fl signal of PAB when added to uPA (sample 4). Likewise, Compound B had little effect on the minor Fl signal of PAB in the presence of PAI-1 (sample 5). Importantly, pretreatment of PAI-1 with Compound B negated the ability of PAI-1 to suppress the augmented Fl signal of PAB in the presence of uPA (sample 6). This result revealed that Compound B (and by extrapolation, PAI-749, its closely-

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related analog) blocked the ability of PAI-1 to occupy the primary specificity pocket of uPA. Binding of the P1 residue in PAI-1 to the primary specificity pocket of the PA is proposed to occur concomitantly with formation of the putative Michaelis-like complex (Ibarra et al, 2004). Consequently, this experiment reveals that the effect of PAI-749 on PAI-1 occurs at the earliest step of the postulated pathway describing the interaction between PAI-1 and the PA (ie. PAI-749 appears to block formation of the reversible Michaelis-like complex between PAI-1 and the PA).

It was previously reported that negatively charged organochemical inactivators of PAI-1 convert PAI-1 to inactive polymers (Pedersen et al, 2003). We thus employed (nondenaturing) BN-PAGE (Schägger et al, 1991) to test if PAI-749 similarly elicited PAI-1 polymerization. PAI-1 was mixed with increasing concentrations of PAI-749 for 60 min and was then subjected to both SDS-denaturing PAGE and BN-PAGE. Treatment of PAI-1 with PAI-749 had no impact on the mobility of PAI-1 as assessed by SDS-PAGE (Fig. 6A). In the absence of PAI-749 treatment, PAI-1 exhibited a tendency towards polymerization when analyzed by BN-PAGE (Fig. 6B, lane 1). With increasing concentrations of PAI-749 there was a dramatic shift in the mobility of PAI-1 to higher molecular species when analyzed by BN-PAGE. The virtual disappearance of PAI-1 in the presence of elevated PAI-749 as assessed by BN-PAGE reflects diffuse migration of the heterogeneous PAI-1 polymer. This assertion is amply supported by cross-reference to panel A showing no diminution of signal when the *same* samples where analyzed by SDS-PAGE. The concentration dependence of the PAI-749 effect on PAI-1 mobility during BN-PAGE (Fig. 6B) was strikingly similar to that of neutralization of PAI-1 activity (Fig. 1) and perturbation of the Fl signal of PAI-NBD119 (Fig. 2B). Hence, all of these effects of PAI-749 on PAI-1 appear to be linked. Interestingly, the mobility of latent PAI-1 during BN-PAGE was unaltered by pretreatment with PAI-749 (data not shown).

When the treatment interval between PAI-1 and PAI-749 was only 5 min (actual exposure time is approximate since there was no quench step prior to BN-PAGE), PAI-1 polymerization was evident but was less extensive (data not shown). The apparent dependence of

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PAI-1 polymerization on exposure time to PAI-749 (5 min vs. 60 min) is reminiscent of the aforementioned impact of exposure time between PAI-1 and PAI-749 on the ability of Tween-80 to "reverse" inactivation of PAI-1 activity (Table 2).

Impact of vitronectin on the ability of PAI-749 to neutralize PAI-1 - Vitronectin (VN) was shown to bind tightly to PAI-1 (Declerck et al, 1988; Wiman et al, 1988). Indeed, PAI-1 exists in plasma largely as a complex with VN. We thus examined the possible impact of VN on the ability of PAI-749 to inhibit PAI-1 activity (Table 4). The addition of VN or BSA (control) to tPA did not alter its activity towards Spectrozyme tPA. As expected, addition of PAI-1 to tPA virtually abolished the catalytic activity of tPA. Pretreatment of PAI-1 with VN or BSA did not suppress its ability to inactivate tPA. Again, as expected, pretreatment of PAI-1 with PAI-749 neutralized the ability of PAI-1 to inhibit tPA. Addition of VN to PAI-1 before the addition of PAI-749 largely blocked the ability of PAI-749 to neutralize PAI-1 inhibitory activity. BSA failed to protect PAI-1 from inactivation by PAI-749. On the other hand, pretreatment of PAI-1 with PAI-749 followed by the addition of VN yielded inactive PAI-1. PAI-1 treated sequentially with PAI-749 and BSA was also largely incapable of inactivating tPA. We conclude that PAI-1 when complexed with VN is shielded from the inhibitory effects of PAI-749. Nonetheless, if PAI-749 neutralizes PAI-1 first, then the subsequent encounter with VN does not reverse the inhibitory effect of the compound.

Impact of PAI-749 on the vulnerability of PAI-1 to plasmin-mediated proteolysis - It was shown previously that plasmin combines with PAI-1 to produce an SDS-stable complex (Reilly et al, 1993). The robust plasmin-generating potential in the vicinity of a thrombus prompted us to explore the effects of PAI-749 on the interaction between plasmin and PAI-1. Addition of plasmin to PAI-1 resulted in formation of small but discernible amounts of the SDS-stable plasmin/PAI-1 complex as shown by SDS-PAGE and protein silver staining (Fig. 7, lane 3). As

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expected, formation of the plasmin/PAI-1 complex was blocked by the presence of PAI-749 (lane 4). Surprisingly, the presence of PAI-749 also triggered the virtual disappearance of the band corresponding to PAI-1 (lane 4). Aprotinin, a potent plasmin inhibitor, blocked the PAI-749 induced disappearance of the PAI-1 protein band (as well as the appearance of the plasmin/PAI-1 covalent complex) (lane 5). PAI-749 did not increase the activity of plasmin towards a chromogenic plasmin substrate (data not shown); hence, the virtual disappearance of PAI-1 in the presence of PAI-749 and plasmin does not appear to be due to a generalized stimulation of plasmin proteolytic activity. The ability of PAI-749 to promote plasmin-mediated degradation of PAI-1 was also evident from data showing a concomitant decrease of residual PAI-1 inhibitory activity (data not shown). Interestingly, latent PAI-1 was refractory to the ability of PAI-749 to promote plasmin-mediated degradation (lanes 7-9). This result corroborated the aforementioned conclusion that PAI-749 neither interacts with latent PAI-1 nor induces its formation.

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DISCUSSION

PAI-749 preserved tPA and uPA activity in the presence of neutralizing amounts of PAI-1. This outcome could arise if (a) PAI-749 binds to PAI-1 and neutralizes its PA-inhibitory activity or (b) PAI-749 binds to the PA and blocks its subsequent interaction with PAI-1. The data presented herein strongly supports the former hypothesis wherein PAI-749 binds directly to PAI-1 and this binding event mediates neutralization of PA inhibitory activity (i.e., PAI-749 is a *bona fide* PAI-1 antagonist). The fact that structurally-related PAI-749 analogs displayed the same rank order of potency for altering the Fl signal of PAI-NBD119 and inhibiting PAI-1 activity firmly established that binding to PAI-1 and antagonism of PAI-1 activity are inextricably linked. While the preponderance of the data points to PAI-749 being a direct inhibitor of PAI-1, it is unresolved why the apparent IC_{50} values of PAI-749 for tPA and uPA are not identical (albeit less than 2-fold different). The apparent ability of the PAI-749 to induce polymerization of PAI-1 could possibly exert disparate effects with respect to neutralization of uPA and tPA.

Elegant studies by Shore and colleagues helped to elucidate the reaction pathway for PAI-1 mediated inhibition of tPA activity (Olson et al, 2001). Insight gleaned from these investigations reveals a number of potential mechanisms by which PAI-749 might neutralize PAI-1. First, PAI-749 could promote the active-to-latent conformational change in PAI-1. Production of the latent state of PAI-1 occurs spontaneously; hence, a compound could bind to PAI-1 and, in turn, decrease the activation energy barrier to formation of the thermodynamically more favorable latent state. Second, PAI-749 could block formation of the initial reversible Michaelis complex between PAI-1 and its target protease. Third, PAI-749 could impede nucleophilic attack of the active site serine of the PA on the reactive site of PAI-1 thereby abrogating formation of the acyl-enzyme intermediate. Fourth, PAI-749 could convert PAI-1 from a suicide inhibitor to a substrate for tPA by facilitating hydrolytic attack on the acyl-enzyme intermediate. The goal of

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this investigation was to determine which of these proposed inhibitory mechanisms is indeed responsible for the ability of PAI-749 to antagonize PAI-1 inhibitory activity.

PAI-749 does not neutralize PAI-1 by inducing formation of latent PAI-1. Support for this conclusion is derived from several different lines of evidence. First, PAI-1 activity is preserved despite limited exposure to inhibitory amounts of PAI-749 (using "Tween-80 sequestration" to reduce the effective PAI-749 exposure prior to subsequent addition of the PA). This result is incompatible with production of the latent form of PAI-1, a stable conformational state that is not reversed upon removal of the triggering agent/condition. Second, the Fl signal of the latent form of PAI-NBD119 either in the presence or absence of PAI-749 differs from that observed when PAI-749 is added to active PAI-NBD119. Third, latent PAI-1 is not susceptible to plasmin-mediated proteolytic degradation in the presence of PAI-749 in stark contrast to active PAI-1 that is treated with PAI-749. Fourth, polymerization of PAI-1 by PAI-749 as shown by BN-PAGE was not seen with latent PAI-1. The results, in sum, clearly established that PAI-749 did not neutralize PAI-1 by triggering formation of latent PAI-1.

PAI-749 appears to exert an inhibitory effect on PAI-1 activity by interfering with the earliest step in the proposed reaction pathway: formation of the Michaelis-like complex between PAI-1 and the PA. The argument rests on two basic premises: (a) Compound B (closely related analog of PAI-749) blocked the ability of PAI-1 to displace PAB from the active site of uPA and (b) the model structure of the Michaelis complex between PAI-1 and tPA based on the crystal structure of the noncovalent *Manduca sexta* serpin 1B-trypsin complex showed that the side chain and amide backbone nitrogen of Arg-346 (PAI-1 P1 residue) are optimally situated in the active site pocket (Ibarra et al, 2004). Hence, displacement of PAB from the PA active site by PAI-1 is predicted to accompany formation of the Michaelis complex. Based on the aforementioned arguments, failure of PAI-749-treated PAI-1 to displace PAB from the PA signifies that the Michaelis complex between PAI-1 and the PA is not produced in the presence of PAI-749. This conclusion is consistent with other experimental observations showing that more distal events in

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the reaction pathway (e.g. formation of the SDS stable complex) have been abolished as well. The most parsimonius explanation for at least one component of the mechanism of action of PAI-749 is that the compound binds directly to PAI-1 and interferes with the ability of PAI-1 to engage in a Michaelis-like complex with tPA.

The ability of low molecular weight PAI-1 antagonists to elicit serpin multimerization was reported previously (Pedersen et al, 2003). PAI-1 polymerization in the presence of PAI-749 was thus was not surprising. However, the impressive potency of PAI-749 at producing this effect is particularly noteworthy. In any event, the PAI-1 polymerization outcome created uncertainty with regards to the mechanism of inhibition of PAI-1 by PAI-749. Namely, was it due to the formation of the binary complex between PAI-1 and PAI-749 or was it due to PAI-749 triggered PAI-1 polymerization? The reversibility studies with Tween-80 shed light on the answer to this key question. Inactivation of PAI-1 by PAI-749 was reversed by Tween-80 when PAI-1 and PAI-749 were allowed to interact for 5 min. However, the Tween-80 induced reversibility was not apparent after 60 min treatment of PAI-1 with PAI-749. We hypothesize that the rapid inhibition of PAI-1 activity by PAI-749 reflects formation of the binary complex (reversed by Tween-80). The subsequent PAI-1 polymerization (not reversed by Tween-80) is not imperative for PAI-1 inactivation but does commit PAI-1 to a pseudo-irreversible state. This interpretation of the data further implies that the rapid change of the Fl signal of PAI-NBD119 by PAI-749 is also probably due to the formation of the binary complex and not the ensuing PAI-1 polymerization. A high likelihood exists that the proximal molecular events following the initial interaction between PAI-1 and PAI-749 display greater complexity with respect to discrete states/conformations of PAI-1. For instance, this possibility is suggested by the apparent biphasic concentration-dependent impact of PAI-749 on the substrate-like behavior of PAI-1 within the bounds of a short (5 min) treatment interval (depicted in Fig. 5). Clearly, while our investigation has provided key mechanistic insight into the action of PAI-749 on PAI-1, there are certain aspects of this interaction that have yet to be elucidated and will require further investigation.

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The ability of PAI-749 to inhibit formation of the plasmin/PAI-1 complex represents another potential profibrinolytic effect of PAI-749; however, the importance of PAI-1 to plasmin neutralization *in vivo* is uncertain due to the vast potential excess of plasmin over PAI-1. An unexpected and potentially more significant finding is that PAI-749 promotes plasmin-mediated degradation of PAI-1. The putative conformational change in PAI-1 (perhaps coincident with formation of serpin multimers) induced by the binding of PAI-749 appeared to expose regions in PAI-1 that are cleaved by plasmin. This PAI-749 mediated effect represents a potential "feed forward" profibrinolytic mechanism. Accordingly, direct PAI-1 antagonism by PAI-749 promotes plasmin formation that, in turn, can lead to greater plasmin production due to plasmin-mediated degradation of the PAI-1/PAI-749 complex. Other PAI-1 antagonists were shown previously to increase the susceptibility of PAI-1 to proteolytic degradation by a variety of proteases such as papain and subtilisin (Einholm et al, 2003). However, plasmin (which was not previously examined) is a particularly relevant protease in light of its high abundance in the vicinity of the thrombus.

The ability of VN to shield PAI-1 from the effects of PAI-749 coupled with the fact that PAI-1 exists predominantly in plasma as a complex with VN might prompt speculation that PAI-749 would not inhibit PAI-1 activity *in vivo*. However, whereas PAI-1 is expressed widely, VN appears to be expressed predominantly by the liver (Seiffert et al, 1994). Hence, "naked" PAI-1 will exist at least transiently after secretion by the source cell and, thus, should be a target for PAI-749 until it combines with VN. The fact that inactivation of PAI-1 activity by PAI-749 is not reversed by subsequent addition of VN is consistent with this hypothesis. Importantly, this proposal is also supported by preclinical *in vivo* experiments in which PAI-749 exerts an impressive antithrombotic effect (J. Hennan et al, manuscript in preparation).

In conclusion, this investigation has uncovered a dual mechanism by which PAI-749 might neutralize PAI-1 activity (Fig. 8). Binding of PAI-749 to PAI-1 blocks the ability of PAI-1 to engage in a complex with the PA. The ensuing increase of PA-mediated plasmin production

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may, in turn, lead to further PAI-749-dependent neutralization of PAI-1 due to proteolytic degradation of the PAI-1 / PAI-749 complex by plasmin. These two PAI-749-mediated effects on PAI-1 should work in concert to elevate tPA and plasmin activities at regions of vascular injury thereby preserving blood vessel patency and contributing to antithrombotic efficacy. Detailed studies of the impact of PAI-749 on PAI-1 activity in blood as well as in the setting of active thrombolysis *in vivo* will be necessary to further explore the clinical antithrombotic potential of this PAI-1 antagonist.

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FOOTNOTE

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FIGURE LEGENDS

Fig. 1: PAI-749 preserved plasminogen activator activity in the presence of PAI-1. PAI-1 was treated with the indicated amount of PAI-749 for 5 min and then mixed with tPA or uPA. Ten min later, plasminogen activator activity was assayed with the respective amidolytic substrate for tPA or uPA. Residual tPA (or uPA) activity (%) is shown. ○, tPA + PAI-1; ● uPA + PAI-1.

Each data point depicts the mean (sem), n=4. The inset shows the structure of PAI-749 [1-Benzyl-3-pentyl-2[6-(1*H*-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1*H*-indole].

Fig. 2: Impact of PAI-749 on the Fl signal of PAI-NBD119, the Fl-tagged PAI-1 variant with NBD at the amino acid 119 position. (A) Spectral scans (excitation: 480 nm; emission: 500-600 nm) of PAI-NBD119 in the absence and presence of increasing concentrations of PAI-749. The PAI-749 concentrations (nM) and associated color-coded scans are shown. (B) Determination of the apparent K_d for PAI-749 binding to PAI-NBD119. The Fl emission signal at 525 nm was used to derive this value. (C) Time-dependent effects of PAI-749 on the Fl signal of PAI-NBD119. 1: PAI-NBD119 (50 nM); 2: PAI-NBD119 (50 nM) + PAI-749 (1 μM). Samples were mixed and the Fl signal was immediately monitored for 300 sec interval (excitation: 480 nm; emission: 525 nm).

Fig. 3: Scatter plot of IC_{50} values (neutralization of PAI-1 activity towards tPA) versus apparent K_d values (perturbation of the Fl signal of PAI-NBD119) for PAI-749 and its closely-related structural analogs. Compounds (PAI-749, B-H) are depicted as filled labeled circles. Linear regression analysis of all data points is shown ($r^2 = 0.65$). The structures of the compounds are identified in Table 1.

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Fig. 4: PAI-749 does not induce formation of the latent form of PAI-1. Active PAI-NBD119 or latent PAI-NBD119 (50 nM) was mixed with DMSO control or PAI-749 (1 μM). After 5 min, the samples were scanned for the Fl signal (excitation: 480 nm; emission: 500-600 nm). The identities of the samples are indicated in the side legend.

Fig. 5. Impact of PAI-749 on the fate of PAI-1 when added to tPA as assessed by SDS-PAGE. (A) PAI-1 (24 nM) was preincubated with PAI-749 for 5 min prior to the addition of tPA (20 nM). After 10 min, the reactions were quenched by the addition of SDS-containing sample prep buffer, fractionated by SDS-PAGE, and proteins were visualized by silver staining. The PAI-749 concentrations (nM) in each sample were 0, 43, 54, 67, 84, 105, 131, 164, 205, 256, 320, 400, and 500 (lanes 1-13, respectively). A mock reaction containing 24 nM PAI-1 without PAI-749 or tPA is shown in lane 14 (B) Densitometry analysis of the formation of covalent complex between tPA and PAI-1 as a function of PAI-749 concentration. For each concentration of PAI-749, pixel intensity of the band corresponding to the serpin-protease complex was normalized as percent total complex observed in absence of PAI-749 (See Materials and Methods).

Fig. 6. PAI-749 promoted PAI-1 polymerization as assessed by BN-PAGE. PAI-1 (20 nM final) was mixed with the indicated concentrations of PAI-749 in HNEPA buffer. After 60 min at room temperature, samples (from the same experiment) were mixed with LDS sample buffer and fractionated by SDS-PAGE (panel A) or mixed with Native Page sample buffer and fractionated by BN-PAGE (panel B). Samples were detected by protein silver staining.

Fig. 7: PAI-749 promotes plasmin-mediated degradation of active PAI-1 but not latent PAI-1. Plasmin, PAI-1 (both active and latent), PAI-749 and aprotinin concentrations were 10 nM, 100 nM, 1 μM and 200 nM, respectively. All reactions were performed at room temperature for 30

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min prior to quenching and fractionation by SDS-PAGE. Lane 1: plasmin; lane 2: active PAI-1; lane 3: active PAI-1 + plasmin; lane 4, active PAI-1 + PAI-749 + plasmin; lane 5, active PAI-1 + PAI-749 + plasmin + aprotinin; lane 6, latent PAI-1; lane 7, latent PAI-1 + plasmin; lane 8, latent PAI-1 + PAI-749 + plasmin; lane 9, latent PAI-1 + PAI-749 + plasmin + aprotinin. Proteins were visualized by silver staining.

Fig. 8: PAI-749 exerts a dual mechanism of action for neutralizing PAI-1 activity. This scheme shows the proposed reaction pathway for the interaction between plasminogen activator inhibitor type I (PAI) and a plasminogen activator (PA). PAI_{latent}: latent PAI; PA•PAI: Michaelis-type complex; PA~PAI: acyl-enzyme intermediate; PAI^{*}: PAI-1 clipped at the reactive site; PA–PAI: SDS-stable covalent complex. The data in this study show that PAI-749 binds directly to PAI-1 and interferes with formation of the Michaelis complex between PAI-1 and the PA. Moreover, PAI-749 upon binding to PAI-1 promotes plasmin-mediated degradation of PAI-1.

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TABLE 1

Assays of PAI-749 and its closely-related analogs

The basic template structure for the PAI-749 chemical series with the 2 variable positions (R_1 and R_2) is depicted. The identity of the substituents in the R_1 and R_2 positions for PAI-749 and Compounds B-H are shown. For each compound, the IC_{50} value using unmodified human PAI-1 (and tPA) and the apparent K_d value using PAI-NBD119 are shown.

	R_1	R_2	**IC_{50} (nM)**	**K_d (nM)**
PAI-749	Benzyl	H	154	253
Compound B	Benzyl	Me	217	446
Compound C	Benzyl	Cl	141	204
Compound D	Benzyl	Br	175	466
Compound E	Methyl	Cl	258	376
Compound F	Methyl	Me	195	367
Compound G	*o*-CF_3-Benzyl	Br	332	580
Compound H	*p*-*t*-Bu-Benzyl	H	180	307

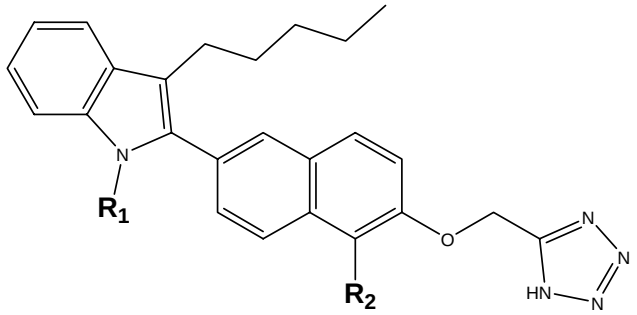

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TABLE 2

Assaying for residual PAI-1 inhibition after limited exposure to PAI-749

Stage I: HNEP buffer alone (samples 1, 4, 7, 10) or HNEP buffer containing 20 nM human PAI-1 (all other samples) was mixed with 1 μM PAI-749 (samples 3, 6, 9, 12) or DMSO vehicle control (all other samples) in HNEP buffer for 5 (samples 1-6) or 60 min (samples 7-12). Stage II: 0.1% Tween-80 was added to samples 4-6 (which sequesters/neutralizes PAI-749 in detergent micelles). Stage III: tPA (20 nM) was added to all samples. After 10 min at room temperature, all samples were mixed with Spectrozyme tPA and residual tPA activity (mOD/min) was assayed. Each value shows the mean values (sd); n=3.

	Stage I	**Stage II**	**tPA activity(mOD/min)**
		0.1% Tween-80	
1	5 min	NO	4.41 (0.52)
2	5 min PAI	NO	0.06 (0.00)
3	5 min PAI + PAI-749	NO	3.24 (0.33)
4	5 min	YES	9.04 (0.62)
5	5 min PAI	YES	0.36 (0.22)
6	5 min PAI + PAI-749	YES	1.12 (0.46)
7	60 min	NO	5.10 (0.01)
8	60 min PAI	NO	0.49 (0.36)
9	60 min PAI + PAI-749	NO	4.36 (0.43)
10	60 min	YES	9.61 (0.99)
11	60 min PAI	YES	0.75 (0.51)
12	60 min PAI + PAI-749	YES	6.41 (0.86)

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TABLE 3

Compound B-Treated PAI-1 does not displace *p*-aminobenzamidine from uPA active site

p-Aminobenzamidine (PAB) added to buffer or buffer containing Compound B served as the background measurements for samples 1-3 and 4-6, respectively. Pretreatments at room temperature (5 min) are shown by grouping of reagents within parentheses. All other additions were placed at room temperature for 5 min prior to analysis of the Fl signal (excitation: 325 nm; emission: 355 nm). Each value shows mean (sem), n=3.

	Samples	**Fl Signal (normalized for PAB alone)**
1	PAB + uPA	27.90(2.35)
2	PAB + PAI	0.05 (0.13)
3	(PAB + uPA) + PAI	0.21 (0.13)
4	(PAB + uPA) + Compound B	24.24 (2.47)
5	PAB + (PAI + Compound B)	2.20 (0.15)
6	(PAB + uPA) + (PAI + Compound B)	24.31 (2.08)

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TABLE 4

Impact of Vitronectin on PAI-749 mediated inactivation of PAI-1

Stage I: HNEPA buffer alone or containing human PAI-1 (24 nM), VN (250 nM), BSA (250 nM) and/or PAI-749 (1 uM) was placed at room temperature for 5 min. Stage II: VN, BSA or PAI-749 were added as indicated; samples were placed at room temperature for 5 min. Stage III: tPA (20 nM) was added to all samples. After 10 min at room temperature, all samples were mixed with Spectrozyme tPA and residual tPA activity (mOD/min) was assayed. Each value shows the mean values (SD); n=3.

	Stage I	**Stage II**	**tPA Activity**
1			9.866 (0.163)
2	VN		11.417 (0.249)
3	BSA		10.630 (0.361)
4	PAI-1		0.098 (0.042)
5	PAI-1 + VN		0.025 (0.028)
6	PAI-1 + BSA		0.186 (0.129)
7	PAI-1 + PAI-749		8.332 (0.069)
8	PAI-1 + VN	+ PAI-749	0.510 (0.110)
9	PAI-1 + BSA	+ PAI-749	9.597 (0.470)
10	PAI-1 + PAI-749	+ VN	10.219 (0.133)
11	PAI-1 + PAI-749	+ BSA	9.486 (0.053)

Figure 1

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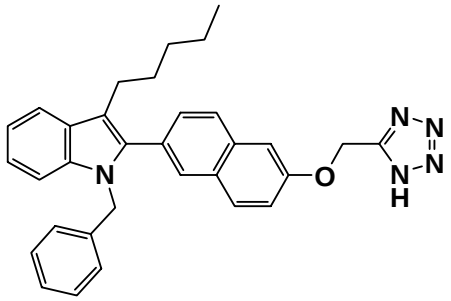

PAI-749

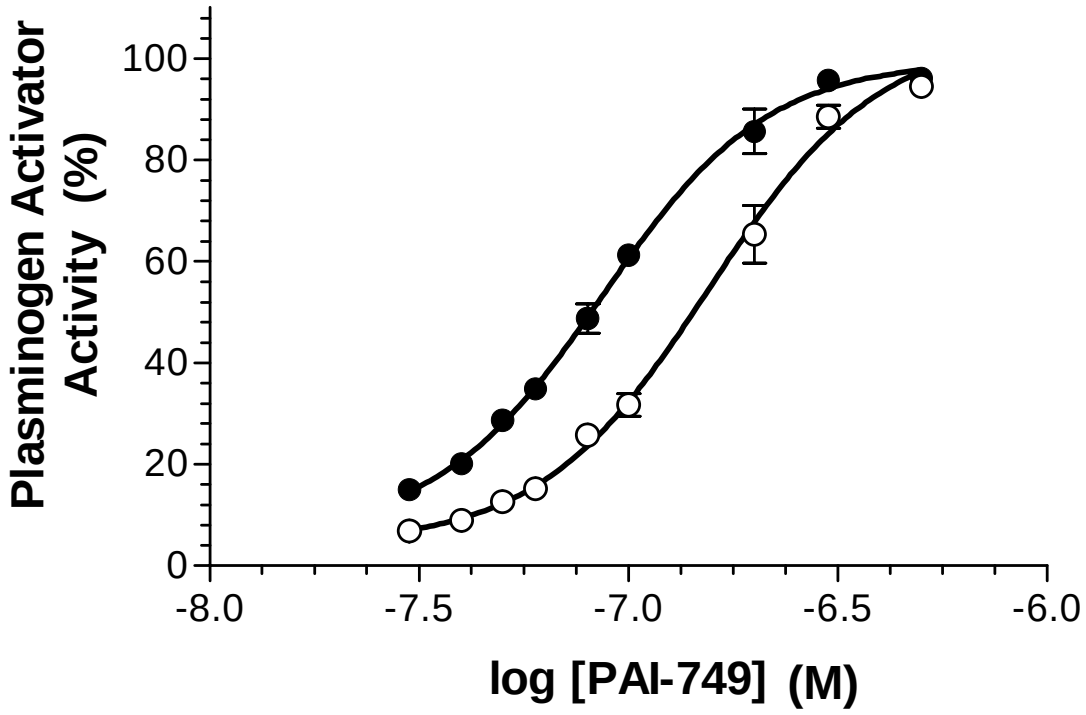

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Figure 2

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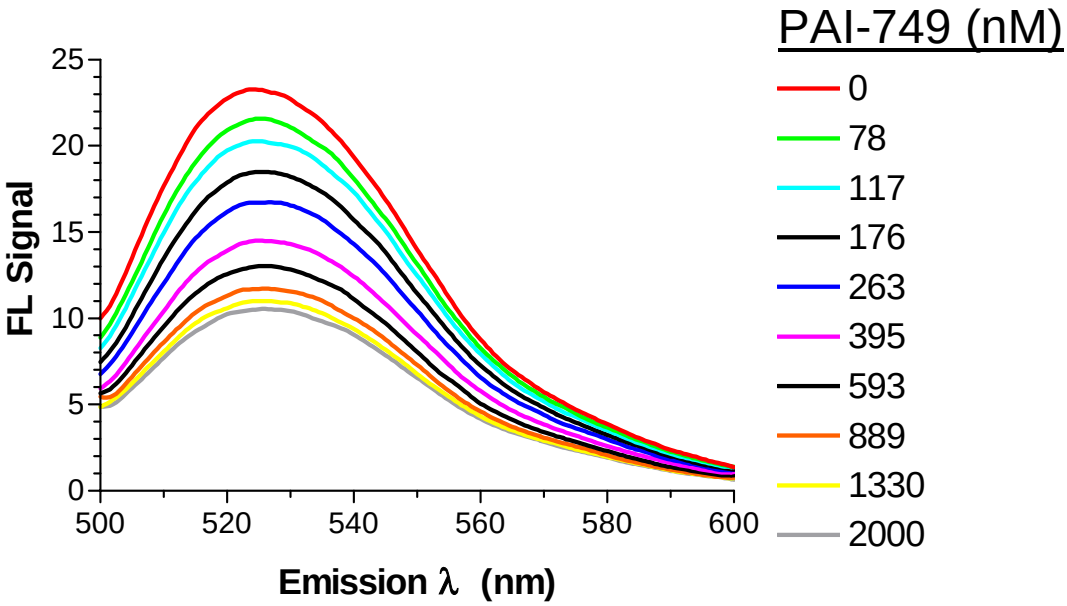

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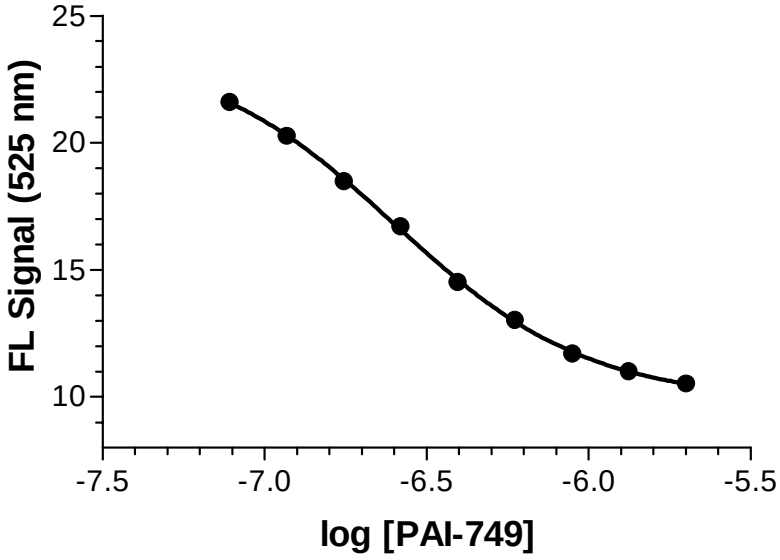

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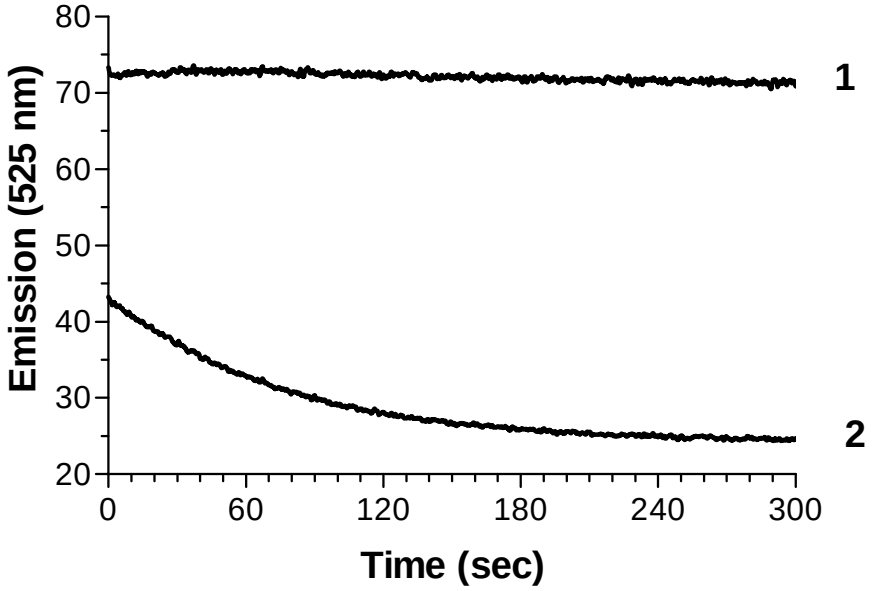

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Figure 3

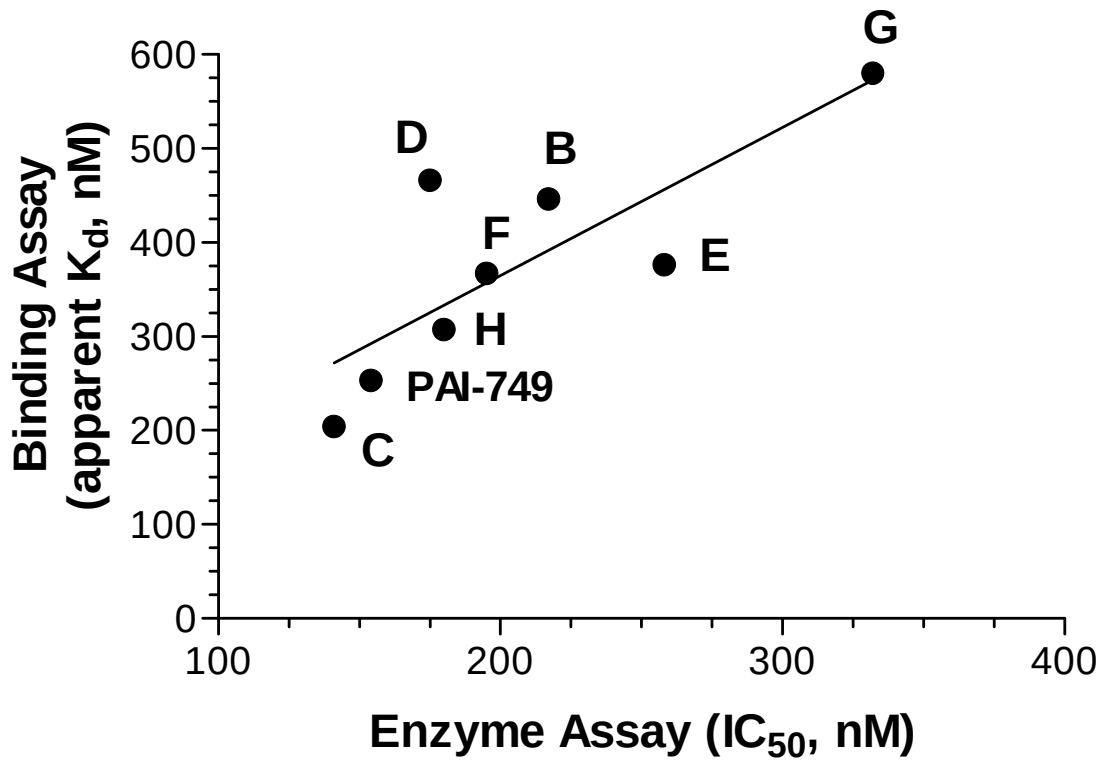

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Figure 4

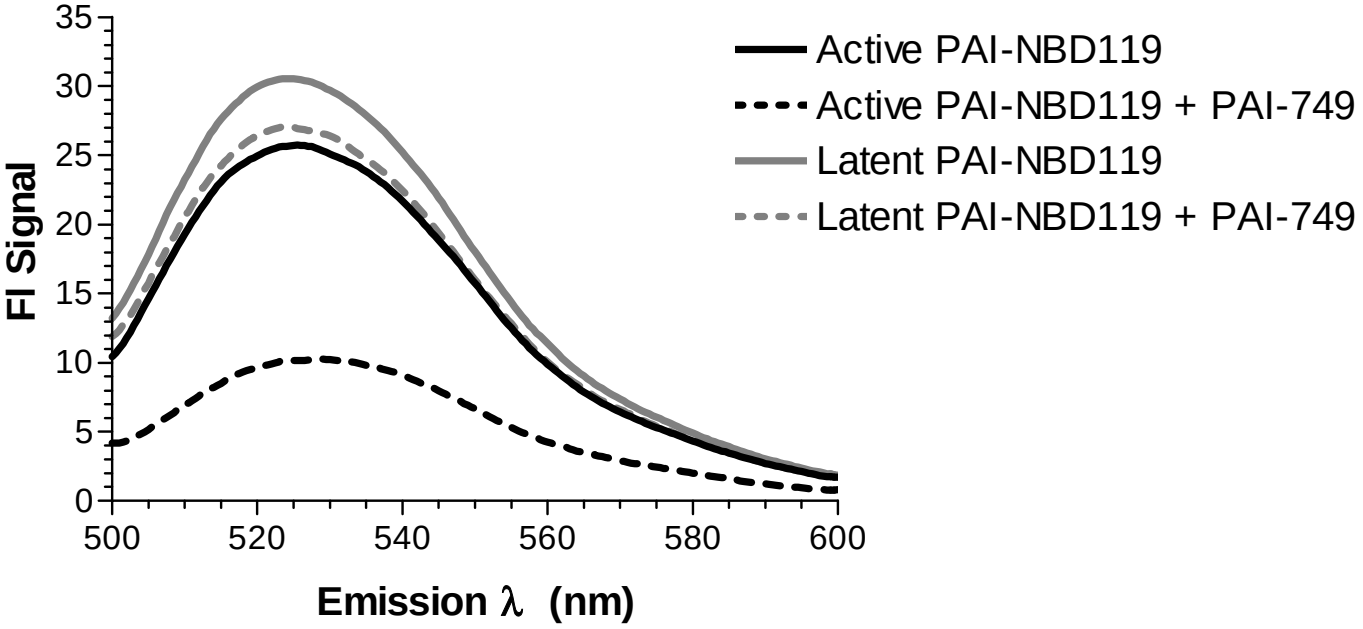

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Figure 5

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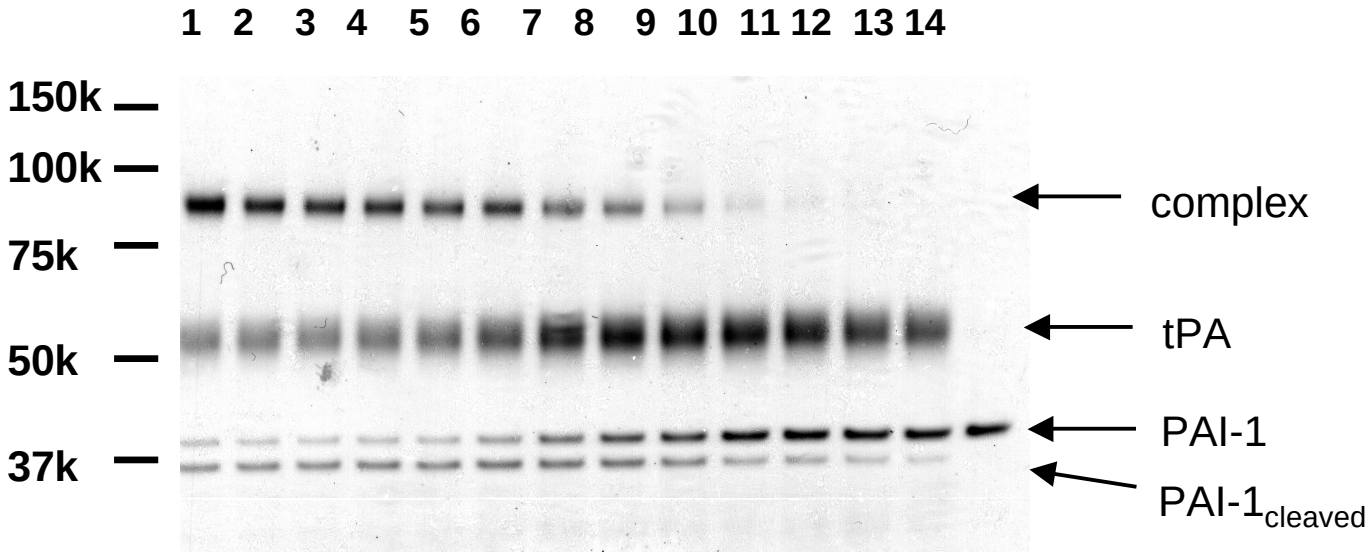

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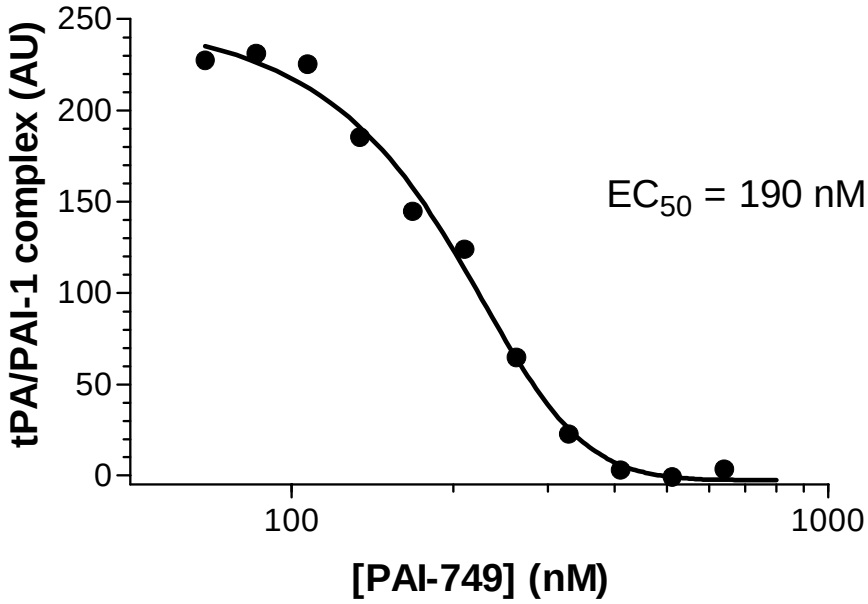

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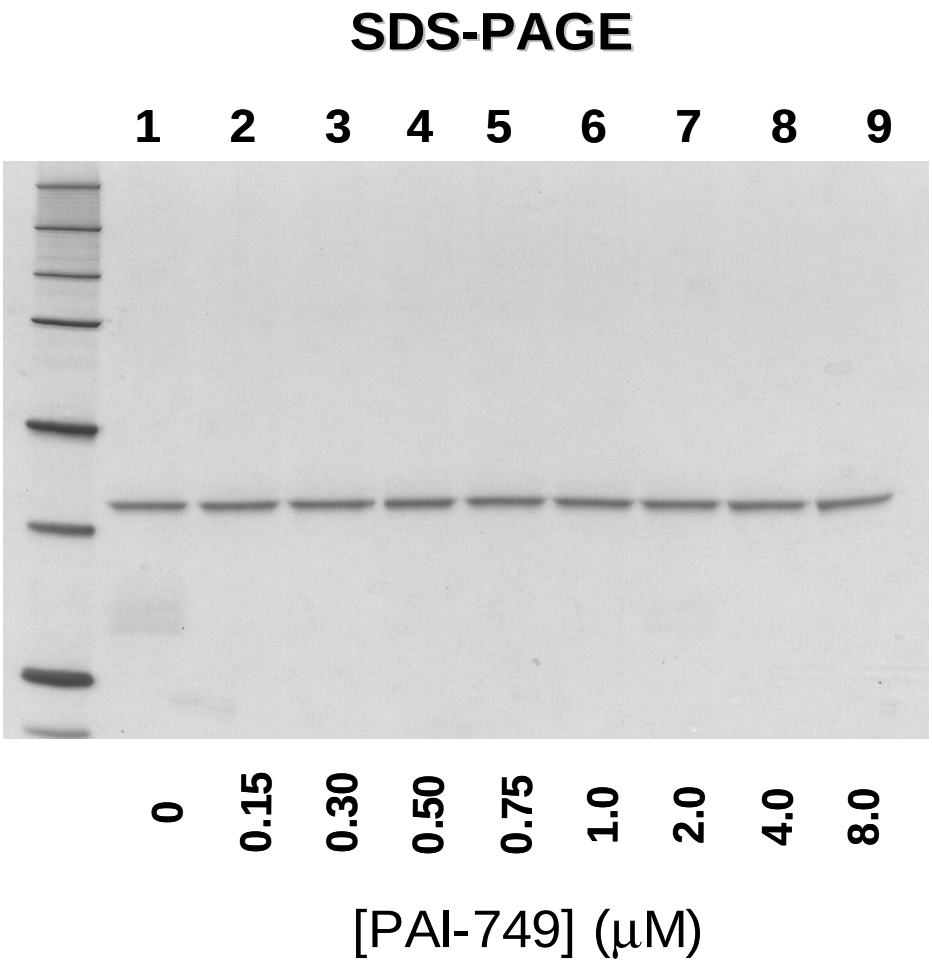
Figure 6

A



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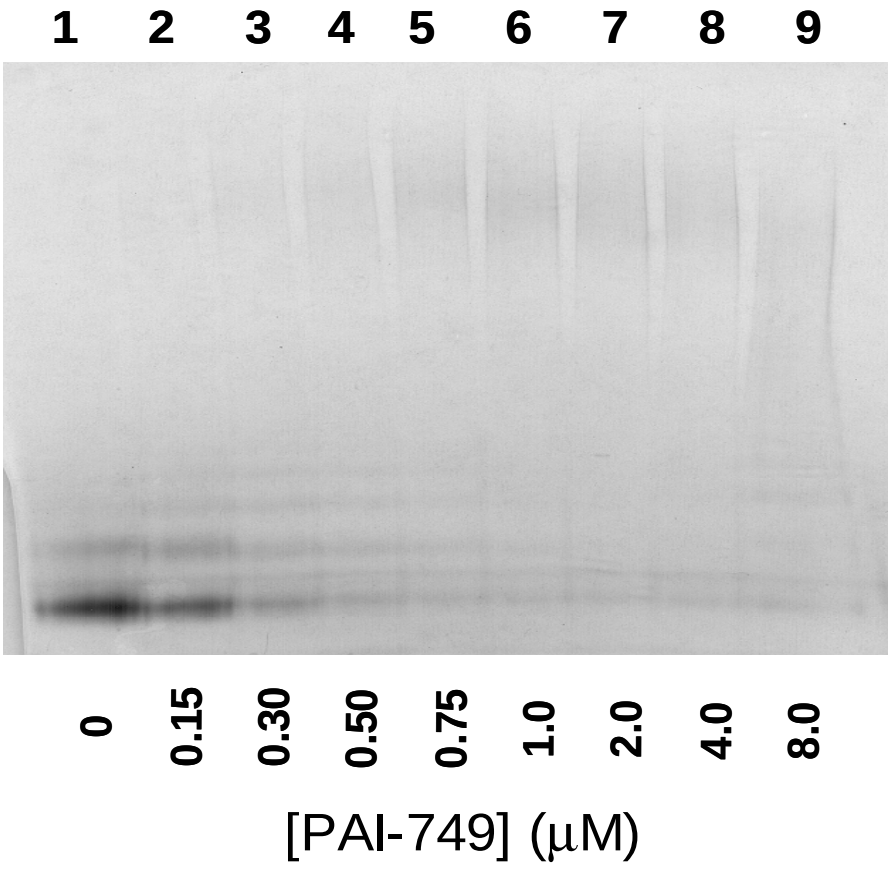

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Figure 7

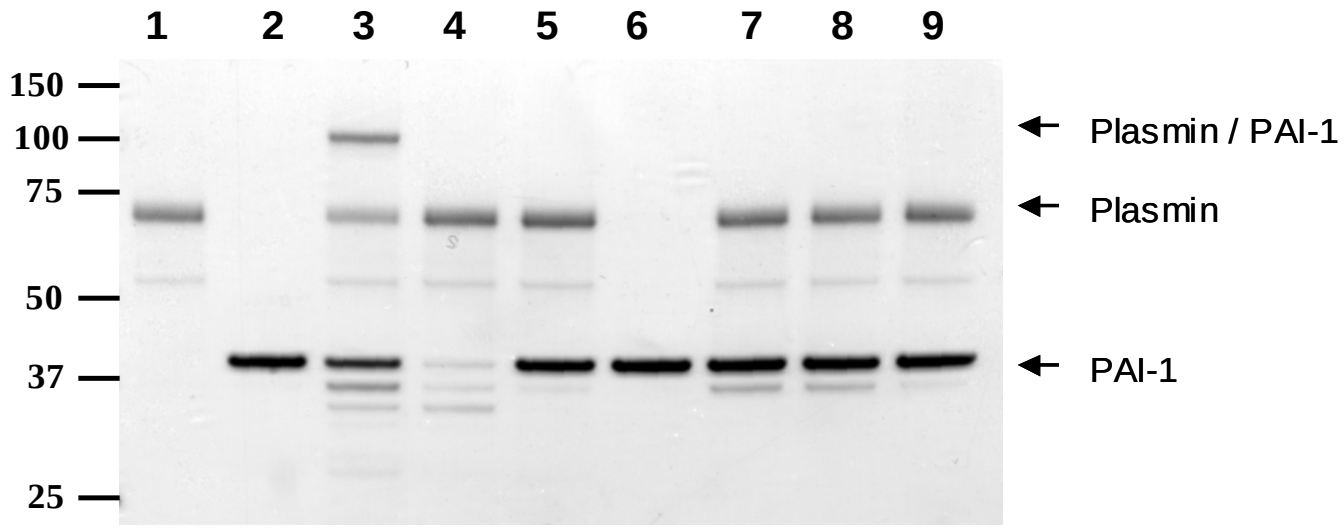

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Figure 8