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Comparative study on the inhibition of plasmin and delta-plasmin via benzamidine derivatives

Nathan J. Alves [a], Jeffrey A. Kline [a, b, *]

[a] Department of Emergency Medicine, Indiana University School of Medicine, Indiana University, Indianapolis, IN 46202, USA
[b] Department of Cellular and Integrative Physiology, Indiana University, Indianapolis, IN 46202, USA

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ABSTRACT

The potent fibrinolytic enzyme, plasmin has numerous clinical applications for recannulizing vessels obstructed by thrombus. Despite its diminutive size, 91 kDa, success in the recombinant expression of this serine protease has been limited. For this reason, a truncated non-glycosylated plasmin variant was developed capable of being expressed and purified from *E. coli*. This mutated plasmin, known as δ-plasmin, eliminates four of the five kringle domains present on native plasmin, retaining only kringle 1 fused directly to the unmodified catalytic domain of plasmin. This study demonstrates that δ-plasmin exhibits similar kinetic characteristics to full length plasmin despite its heavily mutated form; $K_M = 268.78 \pm 19.12$, 324.90 ± 8.43 μM and $K_{cat} = 770.48 \pm 41.73$, 778.21 ± 1.51 1/min for plasmin and δ-plasmin, respectively. A comparative analysis was also carried out to investigate the inhibitory effects of a range of benzamidine based small molecule inhibitors: benzamidine, p-aminobenzamidine, 4-carboxybenzamidine, 4-aminomethyl benzamidine, and pentamidine. All of the small molecule inhibitors, with the exception of unmodified benzamidine, demonstrated comparable competitive inhibition constants (K_i) for both plasmin and δ-plasmin ranging from $K_i < 4$ μM for pentamidine to $K_i > 1000$ μM in the case of aminomethyl benzamidine. This result further supports that δ-plasmin retains much of the same functionality as native plasmin despite its greatly reduced size and complexity. This study serves the purpose of demonstrating the tunable inhibition of plasmin and δ-plasmin with potential applications for the improved clinical delivery of δ-plasmin to treat various thrombi.

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1. Introduction

Acute vascular thrombosis (including: coronary, cerebrovascular, and pulmonary thrombosis) causes more deaths than any other disease process in western society [1]. Blood vessels obstructed by thrombus can be recannulized within hours by enzymatic digestion, mechanical disruption (e.g., angioplasty or catheter disruption) or by a combination of the two methods. In current clinical practice, enzymatic digestion of clots is accomplished by administering an enzyme such as recombinant tissue plasminogen activator [rt-PA], tenecteplase, retaplase, urokinase or streptokinase to activate circulating and clot-bound plasminogen to plasmin [2,3]. Activated plasmin, in turn, cleaves cross-linked γ-chains in the D-domain of fibrin (Aα 148-160) to effectively digest the thrombus [4]. Plasminogen is a 91 kDa zymogen containing 791 amino acids, produced *in-vivo* by the liver, and is heavily glycosylated (2% carbohydrate) in its circulating form (Fig. 1) [5]. When cleaved at Arg561-Val562, plasminogen produces plasmin, a serine protease with a trypsin-like active site (Supporting information Fig. S1). Plasmin binds to thrombi via electrostatic attraction between its five kringle (K) domains to the exposed lysine residues on fibrin with a $K_d = 0.5$ μM for lys-plasmin and $K_d = 5$ μM for glu-plasmin [6]. In order, K4 has the least, K1–K3 have moderate, and K5 has the highest affinity fibrin binding [7,8]. Plasmin's activity is rapidly neutralized in plasma by the circulating proteins α_2-antiplasmin, C1-inhibitor, and macroglobulin [9,10]. The serpin α_2-antiplasmin provides the most rapid and avid inhibition, whereby an Arg-Met residue binds directly to the serine residue in plasmin's active site with a rate constant of 4×10^7 $M^{-1}Sec^{-1}$ [9].

Lack of target specificity poses the largest threat to the clinical therapeutic index of the plasminogen activators. Even when rt-PA is infused directly via a catheter buried within the thrombus, some

* Corresponding author. Department of Emergency Medicine, Department of Cellular and Integrative Physiology, Indiana University, Eskenazi Health, Indianapolis, IN 46202, USA.
E-mail address: jefkline@iu.edu (J.A. Kline).

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degree of systemic plasminogen activation occurs, resulting in fibrinogenolysis and increased bleeding risk [11]. Through recombinant manipulation, a plasmin variant known as delta-plasmin (δ-plasmin), has been produced in which K2–K5 have been deleted from full-length plasmin, while retaining the moderate-affinity of K1 to bind fibrin [12]. Elimination of K2–K5 enables the technical feasibility to synthesize, purify and refold active enzyme from an *E. coli* expression vector. Early clinical trials have demonstrated preclinical and clinical efficacy of catheter-delivered, naked δ-plasmin for recannulizing thrombosed vessels, but at the cost of depleted systemic α_2-antiplasmin [13,14].

The plasmin active site reversibly binds benzamidine containing moieties, in a competitive manner, exhibiting a wide range of inhibition constants (K_i) [15]. This reversible binding property is most commonly utilized for the affinity chromatography purification of activated glu- or lys-plasmin [16]. When occupied by benzamidine, plasmin's active site is shielded from binding by α_2-antiplasmin's Met-Arg sequence preventing the irreversible inhibition of plasmin. Accordingly, benzamidine and its molecular congeners, could function as potential ligands for delivery of δ-plasmin to assist in development of targeted, and longer circulating δ-plasmin based therapeutics. It is for this reason that we sought to characterize the active site binding properties of the following small molecule inhibitors: benzamidine, p-aminobenzamidine, 4-carboxybenzamidine, 4-aminomethyl benzamidine, and pentamidine (Fig. 2) for wild-type (full-length) plasmin, and δ-plasmin by measuring their inhibition constants.

2. Materials and methods

2.1. Materials

Full-length native/wild-type plasmin (purified by affinity chromatography from human plasma), benzamidine, p-aminobenzamidine dihydrochloride, 4-carboxybenzamidine hydrochloride, 4-aminomethyl benzamidine dihydrochloride, and pentamidine isethionate as well as all other organic solvents, small molecules and buffer producing salts were purchased from Sigma–Aldrich (St. Louis, MO). Low binding UV transparent 96-well plates were purchased from Thermo Scientific (Rockford, IL). Lysine Sepharose and Benzamidine Sepharose were purchased from GE Healthcare (Pittsburgh, PA). Chromogenic substrate for plasmin activity tests and determination of kinetic characteristics, S-2251, was purchased from Chromogenix (Orangeburg, NY). Absorbance and fluorescent emission measurements were made using a SpectraMax Plus 96 well plate reader from Molecular Devices (Sunnydale CA). All measurements were carried out in triplicate and data represents means ± standard deviations.

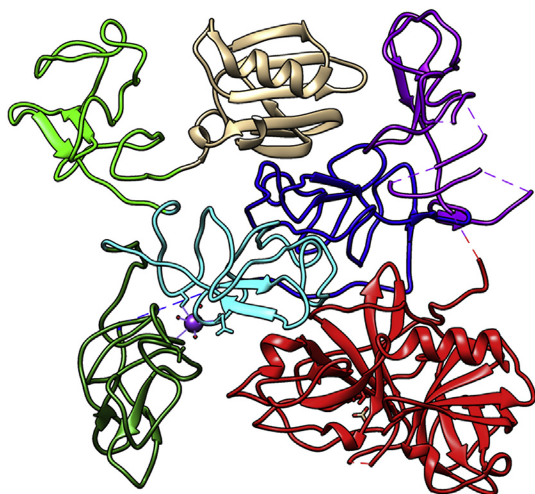

Fig. 1. Crystal structure of full length type II human plasminogen: Highlighted are the different domains including: the pan apple domain (white), Kringle 1–5 (green, cyan, dark green, blue, purple, respectively), and catalytic domain (red), PDB: 4DUR [5]. δ-Plasminogen being comprised only of Kringle 1 (green) and the catalytic domain (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.1.1. δ-Plasmin Production

δ-Plasmin was produced using a modification of the method by Hunt et al. [16]. Briefly, δ-plasminogen zymogen was expressed from a T7 *E. coli* expression system containing the K2–K5 deleted human plasminogen sequence inserted into a pET 24b(+) vector (Novagen; San Diego, CA) between the NdeI and BamHI sites. The δ-plasminogen gene, under lac operon control, was expressed with Isopropyl β-D-thiogalactopyranoside (IPTG). Expressed zymogen was purified with Lysine Sepharose, refolded and activated with streptokinase, and purified by affinity chromatography using Benzamidine Sepharose.

2.2. Extinction coefficient determination

The benzamidine-based inhibitors were precisely weighed and absorbance spectrums were taken from 200 to 350 nm. Absorbance maxima and extinction coefficients were calculated in PBS pH 7.4 at 25 °C and utilized in subsequent binding and inhibition assays.

2.3. Enzyme kinetics

All enzymatic assays were conducted in phosphate buffered saline (PBS, pH 7.4) at 25 °C. The S-2251 assay measures the shift in absorbance from plasmin's cleavage of the chromogenic tag, monitored at 405 nm (Supporting information Fig. S2). K_M, V_{max} and K_{cat} were determined at a fixed concentration of plasmin (1.0 μg/mL) while varying the S-2251 substrate concentrations (0–750 μM) to produce a Lineweaver-Burke plot where the y-intercept = $1/V_{max}$ and slope = K_M/V_{max}. Initial velocities were determined by the slope of the first 60 s of reaction with the S-2251 substrate.

2.4. Inhibition assays

Inhibition assays were carried out at a fixed plasmin concentration in the presence of a range of benzamidine-based inhibitor concentrations from (0–1500 μM) and a minimum of three different S-2251 substrate concentrations ranging from (0–750 μM). K_i values for both native plasmin and δ-plasmin for each small molecule inhibitor were calculated based on the x-axis value at the negative intersection of the inhibition curves at the different S-2251 substrate concentrations utilizing a Dixon Plot.

2.4.1. Fluorescence Titration Dissociation Constant (K_d) Assay

Plasmin was titrated with increasing amounts of p-aminobenzamidine from 0 to 1000 μM in PBS buffer at pH 7.4. By monitoring the change in fluorescence emission (excitation 280 nm, emission at 370 nm) from p-aminobenzamidine associated to plasmin a direct determination of its dissociation constant (K_d) was made [17]. Fitting the data to a sigmoid the K_d value for p-aminobenzamidine to plasmin was determined as the concentration of inhibitor at which half maximum fluorescent emission was achieved.

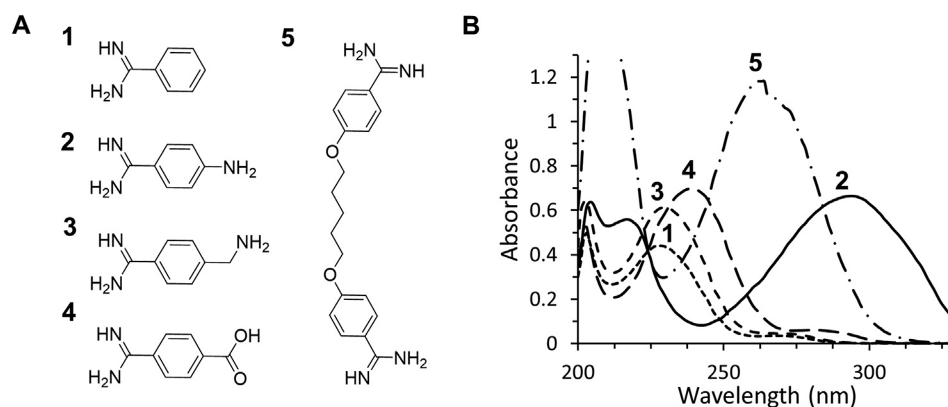

Fig. 2. Benzamidine based small molecule inhibitors: A) 1. benzamidine, 2. p-aminobenzamidine, 3. aminomethyl benzamidine, 4. carboxybenzamidine, and 5. pentamidine. B) Absorbance profiles for the inhibitors in water each at 140 μM demonstrating significant variation in wavelength at maximum absorbance and molar extinction coefficients.

3. Results & discussion

Comparison of full length plasmin and δ-plasmin revealed similar kinetic data. The K_M values were 268.78 ± 19.12, 324.90 ± 8.43 μM and K_{cat} values were 770.48 ± 41.73, 778.21 ± 1.51 min^{-1} for plasmin and δ-plasmin, respectively (Supporting information Fig. S3). These results demonstrate that recombinantly generated, non-glycosylated δ-plasmin is a significantly truncated plasmin variant that maintains similar enzymatic functions as wild-type plasmin.

We sought to characterize a portfolio of benzamidine-based inhibitors of plasmin and δ-plasmin by modifying the chemical species attached to the benzamidine ring to produce varying degrees of enzymatic inhibition. Accordingly, we compared inhibition constants of plasmin and δ-plasmin for benzamidine, p-aminobenzamidine, 4-carboxybenzamidine, 4-aminomethyl benzamidine and pentamidine (Fig. 2A).

Measurement of extinction coefficients for each small molecule inhibitor was necessary to allow for accurate quantification of

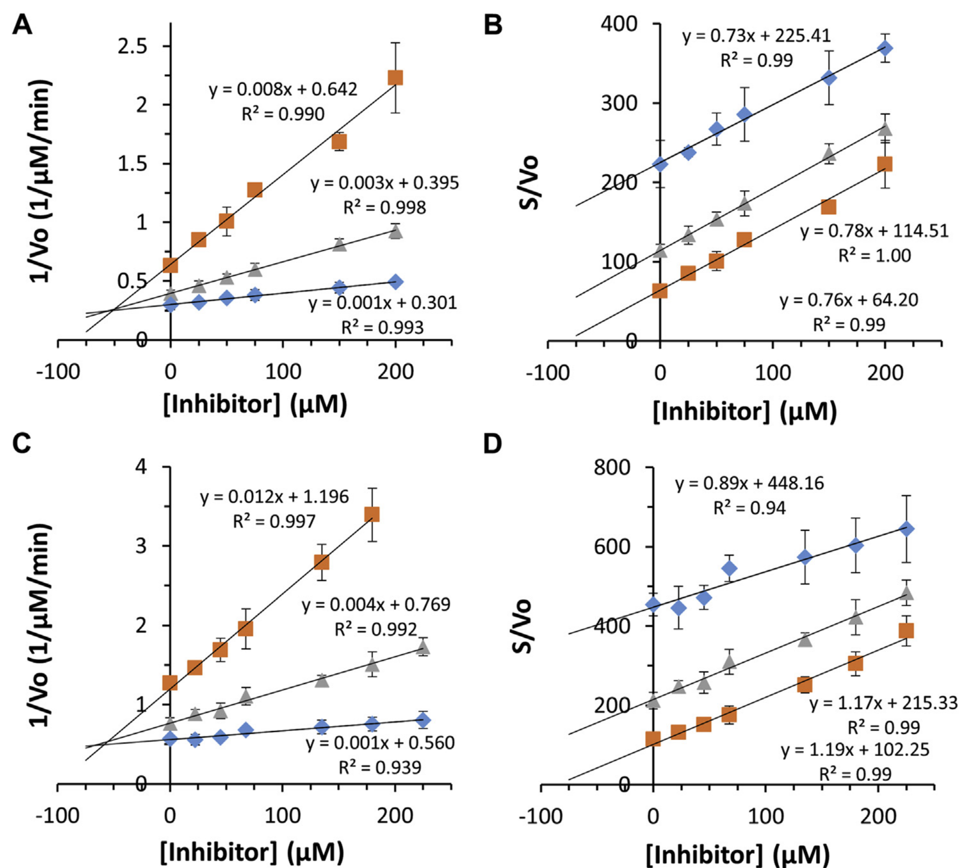

Fig. 3. p-Aminobenzamidine inhibition constant determination: A Dixon Plot analysis was carried out utilizing the S-2251 substrate. p-Aminobenzamidine was incubated with plasmin (A) and δ-plasmin (C) from 0 to 225 μM of inhibitor at a fixed enzyme concentration of 1.0 μg/mL with three different S-2251 concentration of 100 (squares), 350 (triangles), and 750 μM (diamonds) in PBS pH 7.4. The K_i was determined by the negative intersection of the curves demonstrating very similar inhibition constants of 51.9 ± 2.38 and 60.6 ± 6.72 μM for plasmin and δ-plasmin, respectively. S/V_o vs I plots are also shown demonstrating no intersection of the S-2251 curves for plasmin (B) or δ-plasmin (D) indicative of purely competitive inhibition by p-aminobenzamidine. All data represents means (±SD) of triplicate experiments.

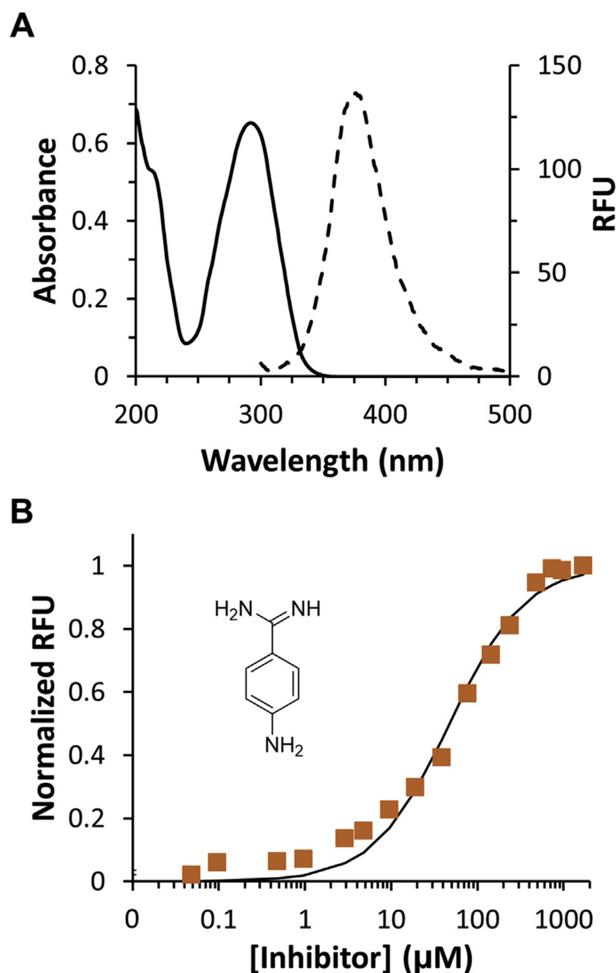

Fig. 4. Binding affinity of p-aminobenzamidine to plasmin: A) Absorbance and fluorescent profiles of p-aminobenzamidine with excitation (solid line) and emission (dashed line) maxima of 294 and 370 nm, respectively. B) K_d determination via fluorescence titration of plasmin with increasing concentrations of p-aminobenzamidine. Binding curve was fit by a sigmoid demonstrating a $K_d = 53.5 \pm 4.42$ μM. All data represents means (±SD) of triplicate experiments.

enzyme inhibition. Each benzamidine variant demonstrated significant differences in absorbance profile, wavelength at maximum absorbance (ABS_{max}) and intensity (Fig. 2B). Extinction coefficients were calculated for each inhibitor based on their ABS_{max} and known inhibitor concentration, as determined by weight (Supporting information Fig. S4). These extinction coefficients were then used in the subsequent plasmin and δ-plasmin inhibition and binding assay (Supporting information Table S1).

The most widely utilized plasmin inhibitor is p-aminobenzamidine, also known as 4-aminobenzamidine, as it is commonly the immobilized ligand used for affinity purification of active plasmin and various other serine proteases through selective binding to its active site [18]. Inhibition assays were carried out at a fixed enzyme concentration in the presence of a range of p-aminobenzamidine concentrations (0–225 μM) and at S-2251 substrate concentrations of 100, 350 and 750 μM [19]. Utilizing a Dixon Plot analysis the K_i values for both plasmin and δ-plasmin were very similar at 51.9 ± 2.38 and 60.6 ± 6.72 μM, respectively (Fig. 3A and C). The Dixon Plot analysis on its own is not sufficient to accurately differentiate between competitive, uncompetitive, non-competitive or mixed modes of inhibition. For this reason an additional analysis of the data plotted as S/V_o vs inhibitor concentration was carried out. Taken together, the intersection of the S-2251 assay curves on the Dixon Plot above the x-axis in conjunction with the lack of an intersection of the S-2251 curves on the S/V_o vs I plot indicates that inhibition of both plasmin and δ-plasmin by p-aminobenzamidine is purely competitive inhibition (Fig. 3B and D) [20]. The very similar K_i values demonstrate the conserved nature of the active site accessibility by benzamidine congeners to both the native and truncated forms of plasmin.

p-Aminobenzamidine's fluorescent properties enable the implementation of a fluorescent titration assay to directly determine its binding affinity to plasmin. Upon binding to plasmin the p-aminobenzamidine exhibits a unique change in fluorescence that can be used to determine its dissociation constant (K_d) [17]. At a constant plasmin concentration, increasing amounts of p-aminobenzamidine were titrated into the sample monitoring the fluorescence emission at 370 nm (Fig. 4A). The K_d of p-aminobenzamidine for plasmin, based on the fluorescent titration assay, was 53.5 ± 4.42 μM (Fig. 4B) compared to the K_i value of 51.9 ± 2.38 μM determined via the S-2251 inhibition assay. This orthogonal method of determining affinity of p-aminobenzamidine for the plasmin's active site in the absence of the S-2251 substrate functions to validate the K_i values determined via the Dixon Plot analysis as K_i and K_d are equal in the case of purely competitive enzymatic inhibition. None of the other benzamidine-based inhibitors in Fig. 2A exhibited this characteristic and were therefore not amenable to a similar fluorescent titration assay.

Inhibition constants for the remaining small molecule inhibitors with plasmin and δ-plasmin were carried out utilizing the S-2251 substrate with the results summarized in Table 1. Both plasmin and δ-plasmin demonstrated similar inhibition constants across most of the small molecule inhibitors including: p-aminobenzamidine, aminomethyl benzamidine (both very weak inhibition, $K_i > 1000$ μM), carboxybenzamidine, and pentamidine (Fig. 3 and Supporting Information Figs. S5–S7, respectively). In all instances, the benzamidine derivatives demonstrated a competitive inhibition of both native and δ-plasmin as indicated by no intersection of the S-2251 curves when plotted as S/V_o vs inhibitor concentration (Supporting information Fig. S5–S8). The inhibition constants for δ-plasmin were only slightly higher, (range +3 to +82%) indicating weaker inhibition. This is likely the result of increased flexibility of the globular protein structure caused by the removal of four of the five kringle domains indirectly impacting the native conformation of the active site. Of note, the K_i values determined for unmodified benzamidine exhibited the greatest deviation (5-fold) from plasmin to δ-plasmin of 32.23 ± 3.02 and 160.78 ± 11.72 μM, respectively (Supporting information Fig. S8). The uncharacteristically wide variation in K_i observed with unmodified benzamidine (+503%) was likely due to the smaller size of benzamidine compared to the other small molecule inhibitors allowing for more degrees of freedom for binding orientations within the catalytic domain of the more flexible δ-plasmin resulting in the largest K_i variation between the two plasmin variants.

Pentamidine, an FDA approved drug for the treatment of various microbial and parasitic infections, and the largest of the small

Table 1
Inhibition constants: K_i values for all small molecule inhibitors tested comparing plasmin to δ-plasmin.

Inhibitor Molecules	Plasmin K_i (μM)	δ-Plasmin K_i (μM)
Benzamidine	32 ± 3.0	161 ± 11.7
p-Aminobenzamidine	51 ± 2.4	61 ± 6.7
4-Carboxybenzamidine	292 ± 6.5	301 ± 19.2
Aminomethyl benzamidine	1074 ± 18.7	1408 ± 67.2
Pentamidine	2.2 ± 0.50	4.0 ± 1.0

molecules, exhibited the strongest inhibition of plasmin and δ-plasmin with K_i values <4 μM. These K_i values represent a >15-fold stronger inhibition than the next best inhibitor for both plasmin and δ-plasmin. Since pentamidine is essentially two benzamidine moieties linked together it would be expected that the K_i values would be approximately 2-fold better than the best inhibitor. The 13-fold discrepancy demonstrates that inhibition is more than simply the arithmetic sum of the number of benzamidine moieties on the molecule and rather there is also a strong avidity effect present resulting in a much stronger inhibition of plasmin due to the multivalent presentation of benzamidine [21,22]. The close proximity of the two benzamidine moieties allows for an increased probability of plasmin rebinding resulting in an apparent increase in the strength of inhibition. This avidity phenomena can be exploited to improve inhibition constants for other inhibitors by simply increasing their valency and may provide a means of tuning inhibition for the therapeutic delivery of δ-plasmin.

These data show that a recombinantly produced, truncated and non-glycosylated form of plasmin, maintains comparable kinetic parameters and responds very similarly to small molecule inhibitors compared with native plasmin. The results support the development of methods to control the degree of inhibition of δ-plasmin through selection of a monovalent small molecule inhibitor as well as increasing valency to take advantage of a multivalent avidity affect to facilitate increased control over the direct delivery of δ-plasmin for clinical use in the enzymatic digestion of thrombi.

Conflict of interest

Both authors are inventors on a provisional US patent involving the subject matter.

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The authors would like to thank Grifols for supplying us with the δ-plasmin producing *E. coli* bacterial strain and Daren Beam MD for producing the δ-plasmin in our laboratory.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2014.12.117.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2014.12.117.

References

[1] S.L. Murphy, J. Xu, K.D. Kochanek, Deaths: final data for 2010. National vital statistics reports : from the centers for disease control and prevention, National Center for Health Statistics, Natl. Vital Stat. Syst. 61 (2013) 1–117.

[2] K. Ouriel, E.L. Welch, C.K. Shortell, K. Geary, W.M. Fiore, C. Cimino, Comparison of streptokinase, urokinase, and recombinant tissue-plasminogen activator in an in-vitro model of venous thrombolysis, J. Vasc. Surg. 22 (1995) 593–597.

[3] R. Fears, M.J. Hibbs, R.A.G. Smith, Kinetic-studies on the interaction of streptokinase and other plasminogen activators with plasminogen and fibrin, Biochem. J. 229 (1985) 555–558.

[4] L. Medved, W. Nieuwenhuizen, Molecular mechanisms of initiation of fibrinolysis by fibrin, Thromb. Haemost. 89 (2003) 409–419.

[5] R.H.P. Law, T. Caradoc-Davies, N. Cowieson, A.J. Horvath, A.J. Quek, J.A. Encarnacao, D. Steer, A. Cowan, Q. Zhang, B.G.C. Lu, R.N. Pike, A.I. Smith, P.B. Coughlin, J.C. Whisstock, The X-ray crystal structure of full-length human plasminogen, Cell Reports 1 (2012) 185–190.

[6] M.A. Lucas, L.J. Fretto, P.A. Mckee, The binding of human-plasminogen to fibrin and fibrinogen, J. Biol. Chem. 258 (1983) 4249–4256.

[7] V.V. Novokhatny, Y.V. Matsuka, S.A. Kudinov, Analysis of ligand-binding to kringles-4 and kringles-5 fragments from human-plasminogen, Thromb. Res. 53 (1989) 243–252.

[8] S.G. Mccance, N. Menhart, F.J. Castellino, Amino-acid-residues of the kringle-4 and kringle-5 domains of human plasminogen that stabilize their interactions with omega-amino acid ligands, J. Biol. Chem. 269 (1994) 32405–32410.

[9] P.K. Anonick, B.B. Wolf, S.L. Gonias, Regulation of plasmin, miniplasmin, and streptokinase-plasmin complex by alpha-2-antiplasmin, alpha-2-macroglobulin, and antithrombin-iii in the presence of heparin, Thromb. Res. 59 (1990) 449–462.

[10] H.S. Cummings, F.J. Castellino, Interaction of human plasmin with human alpha-2-macroglobulin, Biochemistry (N.Y.) 23 (1984) 105–111.

[11] T. Enden, Y. Haig, N. Klow, C. Slagsvold, L. Sandvik, W. Ghanima, G. Hafsahl, P.A. Holme, L.O. Holmen, A.M. Njaastad, G. Sandbaek, P.M. Sandset, CaVenT study Grp. Long-term outcome after additional catheter-directed thrombolysis versus standard treatment for acute iliofemoral deep vein thrombosis (the CaVenT study): a randomised controlled trial, Lancet 379 (2012) 31–38.

[12] V.J. Marder, V. Novokhatny, Direct fibrinolytic agents: biochemical attributes, preclinical foundation and clinical potential, Thromb. Haemost. 8 (2010) 433–444.

[13] V.J. Marder, S. Manyak, T. Gruber, A. Goyal, G. Moreno, J. Hunt, J. Bromirski, P. Scuderi, S.R. Petteway Jr., V. Novokhatny, Haemostatic safety of a unique recombinant plasmin molecule lacking kringles 2–5, Thromb. Haemost. 104 (2010) 780–787.

[14] V.J. Marder, A.J. Comerota, R.D. Shlansky-Goldberg, J.P. Davis, C. Deng, K. Hanna, D. Fineberg, Safety of catheter-delivered plasmin in patients with acute lower extremity arterial or bypass graft occlusion: phase I results, Thromb. Haemost. 10 (2012) 985–991.

[15] F. Markward, H. Landmann, P. Walsmann, Comparative studies on inhibition of trypsin, plasmin and thrombin by derivatives of benzylamine and benzamidine, Eur. J. Biochem. 6 (1968) 502.

[16] J.A. Hunt, S.R. Petteway Jr., P. Scuderi, V. Novokhatny, Simplified recombinant plasmin: production and functional comparison of a novel thrombolytic molecule with plasma-derived plasmin, Thromb. Haemost. 100 (2008) 413–419.

[17] S.A. Evans, S.T. Olson, J.D. Shore, Para-aminobenzamidine as a fluorescent-probe for the active-site of serine proteases, J. Biol. Chem. 257 (1982) 3014–3017.

[18] G. Schmer, Purification of bovine thrombin by affinity chromatography on benzamidine-agarose, H-S Z Physiol. Chem. 353 (1972) 810.

[19] A.Z. Budzynski, Chromogenic substrates in coagulation and fibrinolytic assays, Lab. Med. 32 (2001) 365–368.

[20] A. Cornish-Bowden, A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors, Biochem. J. 137.1 (1974) 143–144.

[21] M.J. Cloninger, B. Bilgicer, L. Li, S.L. Mangold, S.T. Phillips, M.L. Wolfenden, Multivalency, in: Anonymous Supramolecular Chemistry, John Wiley & Sons, Ltd, 2012, pp. 95–115.

[22] V.M. Krishnamurthy, L.A. Estroff, G.M. Whitesides, Multivalency in ligand design, in: W. Jahnke, D.A. Erlanson (Eds.), Fragment-based Approaches in Drug Discovery, Wiley-VCH, Weinheim, 2006, pp. 11–53.