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

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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.

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

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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.

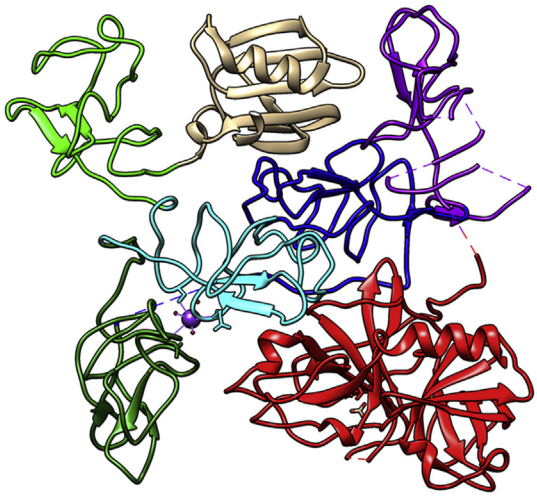

Fig. 1. Crystal structure of full length type II human plasminogen: Highlighted are the different domains including: the pan apple domain (white), Kringle 1–5 (green, cyan, dark green, blue, purple, respectively), and catalytic domain (red), PDB: 4DUR [5]. δ-Plasminogen being comprised only of Kringle 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.