Research Article
doi.org/10.1002/cmdc.202200364
Chemistry Europe
European Chemical Societies Publishing

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Multivalent Benzamidine Molecules for Plasmin Inhibition: Effect of Valency and Linker Length

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There is an emerging interest in utilizing synthetic multivalent inhibitors that comprise of multiple inhibitor moieties linked on a common scaffold to achieve strong and selective enzyme inhibition. As multivalent inhibition is impacted by valency and linker length, in this study, we explore the effect of multivalent benzamidine inhibitors of varying valency and linker length on plasmin inhibition. Plasmin is an endogenous enzyme responsible for digesting fibrin present in blood clots. Monovalent plasmin(ogen) inhibitors are utilized clinically to treat hyperfibrinolysis-associated bleeding events. Benzamidine is a reversible inhibitor that binds to plasmin's active site. Herein, multivalent benzamidine inhibitors of varying valencies (mono-, bi- and tri-valent) and linker lengths (~1–12 nm) were synthesized to systematically study their effect on plasmin inhibition. Inhibition assays were performed using a plasmin substrate (S-2251) to determine inhibition constants (Ki). Pentamidine (shortest bivalent) and Tri-AMB (shortest trivalent) were the strongest inhibitors with Ki values of 2.1 ± 0.8 and 3.9 ± 1.7 μM, respectively. Overall, increasing valency and decreasing linker length, increases effective local concentration of the inhibitor and therefore, resulted in stronger inhibition of plasmin via statistical rebinding. This study aids in the design of multivalent inhibitors that can achieve desired enzyme inhibition by means of modulating valency and linker length.

Introduction

Multivalency (multivalent avidity) is defined as the enhanced response obtained with multiple binding ligands linked on a common scaffold compared to the total response observed with an equivalent number of monovalent ligands.[1] Velcro is a common synthetic material that macroscopically portrays multivalency in which numerous weak loop and hook interactions result in an overall strong association between the two surfaces.[2] There is an emerging interest in using multivalency for medicinal chemistry applications, especially enzyme inhibition, as strong and selective inhibitors can be derived from ligands that have low affinity and low selectivity leveraging multivalency.[3] Multivalent inhibitors for enzyme inhibition is relatively a new concept with much of the research having been performed in glycosidases and carbonic anhydrases to achieve stronger inhibition and improved isoform selectivity.[4,5]

In this study, we aim to understand the effect of valency and linker length on inhibition of plasmin by multivalent benzamidine molecules. Plasmin is a member of the large serine protease family to which one-third of all known proteases belong to.[6] Benzamidine inhibits a variety of serine proteases that include plasmin, trypsin, thrombin. Therefore, understanding the effect of valency and linker length of these multivalent benzamidine molecules will not only assist in design and synthesis of potent plasmin inhibitors but also in design of inhibitors for other trypsin-like serine proteases.[7] In addition, the outcomes from this study can also be extended to achieve desired inhibition of proteases or enzymes that belong to other families.

Multivalency is a prevalent mechanism in nature used to achieve strong and selective, yet reversible, binding.[8] Interactions between E. Coli and urethral endothelial cells, transcription factors and DNA, hemagglutinin (HA) on influenza virus and sialic acid (SA) on bronchial epithelial cells are all naturally occurring examples of multivalency.[9] Multivalency can significantly enhance overall binding. For instance, weak singular HA–SA interactions with association constants of $10^3\ M^{-1}$ have multivalent avidity of $\sim 10^{13}\ M^{-1}$, that is comparable to the strongest monovalent interaction of biotin–streptavidin ($10^{15}\ M^{-1}$). Unlike monovalent interactions that have only two binding modes, binding or no binding, multivalent interactions can provide many different binding modes ranging from all inhibitor moieties being bound, some bound and some unbound, and all unbound.[10] Multivalency gives the ability to tune overall binding avidity simply by modifying the valency ("n", number of ligands or inhibitor moieties) of the binding molecule.[11] Besides valency, linker length, flexibility, shape and orientation of the multivalent inhibitor are a few other parameters that are known to influence multivalent inhibition.[12]

Multivalent polymers, dendrimers, proteins, and liposomes have been utilized as pathogenic targets and were shown to be more effective by orders of magnitude than their monovalent versions.[13,14] Enhanced binding through multivalency can be

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmdc.202200364

achieved by four different mechanisms: (a) statistical rebinding: close proximity of multiple moieties promotes rebinding; (b) chelate effect: multiple moieties simultaneously bind to multiple active sites; (c) subsite binding: interactions with both active and non-active domains or multiple interactions with non-active domains; (d) clustering: molecular interaction across multiple enzymes.[1,3] Herein, we propose to use multivalent benzamidine derivatives of varying valency and linker length to determine their effect on the statistical rebinding mechanism of multivalent plasmin inhibition (Figure 1).

Plasmin is the only *in vivo* fibrinolytic enzyme that is responsible for degrading fibrin in blood clots to achieve clot lysis.[15] It is activated from its precursor plasminogen and possesses a light chain (~25 kDa) with the active site and a heavy chain (~60 kDa) comprising 5 kringle domains.[16] Plasmin inhibitors are useful for treating hyperfibrinolysis associated bleeding in trauma or surgeries.[17] Tranexamic Acid (TXA) and ε-Aminocaproic Acid (EACA) are lysine analogues that are clinically used as antifibrinolytic agents to resolve hyperfibrinolysis associated bleeding. However, these agents do not inhibit plasmin directly but instead inhibit plasminogen activation and plasmin(ogen)-fibrin interactions.[18,19] As plasmin plays a vital role not only in hemostasis but also in immune, and inflammatory responses, active site plasmin inhibitors that inhibit plasmin directly, are useful for treating cancer and inflammation in addition to bleeding disorders.[20,21] Moreover, active site plasmin inhibitors are also expected to be more rapid and effective in reducing bleeding.[22]

Various plasmin inhibitors that comprise of cyclohexanone/cyclohexane, guanidine/amidine, quinidine, tripeptides with nitrile war heads, reactive aldehyde peptidomimetics, cyclic peptidomimetics, polypeptides of the Kunitz and Kazal-type have been studied.[23] Of these, inhibitors containing benzamidine, benzylamine, tranexamic acid and lysine are the most potent small molecule inhibitors of plasmin(ogen). However, in these strategies, the utilization of multivalency has been limited and is largely applied to achieve subsite binding.[24] Benzamidine and its derivatives are common reversible, competitive inhibitors of trypsin family proteases that bind to the active site of plasmin via an amidine group.[25,26]

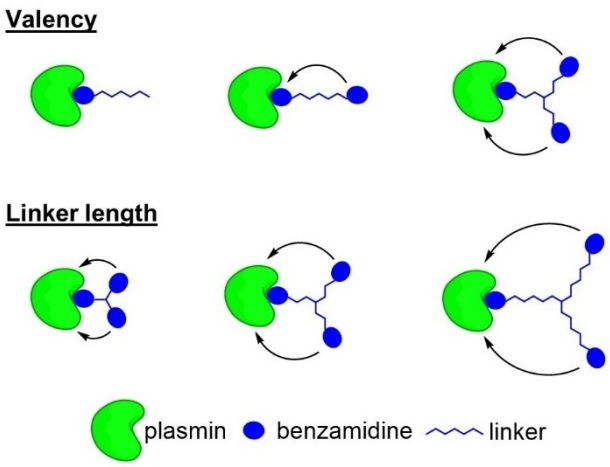

Figure 1. Varying valency and linker length contribute to multivalent inhibition of plasmin by benzamidine via statistical rebinding.

Alves *et al.* have shown that pentamidine, an FDA approved bivalent benzamidine, was a 13-fold stronger plasmin inhibitor than the strongest monovalent benzamidine owing to multivalent avidity effects, presumably statistical rebinding as pentamidine is a short inhibitor (0.9 nm).[27] Herein, we propose to further explore the effect of multivalency by studying the effect of multivalent inhibitors of varying valencies and linker lengths on plasmin inhibition via statistical rebinding.

Results and Discussion

Inhibitor design and synthesis: To explore multivalent inhibition of plasmin by benzamidine derivatives, monovalent (m-dPEGx–AMB; x = 2, 4, 12, and 24), bivalent (Bis-dPEGx–AMB; x = 2, 5, 13, and 25) and trivalent benzamidine (Tri-dPEGx–AMB; x = 0, 4, 8, and 12) inhibitors of separation lengths between benzamidine moieties ranging from ~1–12 nm were synthesized. For this, 4-aminomethyl benzamidine (AMB) and polyethylene glycol (PEG) linkers were conjugated using amine reactive n-Hydroxysuccinimide (NHS) chemistry (Figure 2A). The inhibitors were purified using reverse phase High Performance Liquid Chromatography (HPLC) and confirmed by mass spectrometry. HPLC Chromatogram and mass spectrum for Bis-dPEG2–AMB are shown as exemplary data in Figure 2B and 2 C (see Supporting Information Figures S1–S12 for additional synthesis details). Monodisperse PEG linkers purchased from Quanta BioDesign were used for synthesis to ensure uniform distribution and to have precise control of the linker length to isolate its effects on inhibition. As linker length dictates the mechanism of multivalent inhibition, multivalent benzamidines of lower valency that have short and flexible PEG linkers were specifically synthesized to promote statistical rebinding and minimize clustering and other modes of multivalent inhibition.[28,1]

Benzamidine has also been shown to exhibit weak subsite binding to the light chain and kringle 5 of plasmin.[29] However, prior comparison of benzamidine inhibition across both plasmin and delta-plasmin, a recombinant plasmin variant possessing only the kringle 1 domain and active site, demonstrates that the active site is the primary benzamidine binding site and therefore, subsite binding impact has minimal effect.[27]

Inhibition assays: Inhibition assays were performed for all synthesized inhibitors, free AMB, and Pentamidine to determine their inhibition constants (K_i). The inhibitors are shown to scale relative to plasminogen (PDB ID: 4DUR) in Figure 3 and their separation lengths between benzamidine moieties are reported in Table 1.[30] K_i values represent the potency of the inhibitors and were determined using Dixon plot analysis. A smaller K_i value indicates stronger inhibition. K_i values of mono-, bi-, and trivalent inhibitors ranged from 259.4–1,395 μM, 2.1–290.4 μM, and 3.9–241.9 μM, respectively (Table 1). Cornish-Bowden plots determined the inhibition to be competitive for all inhibitors as indicated by parallel lines. Dixon and Cornish-Bowden plots for

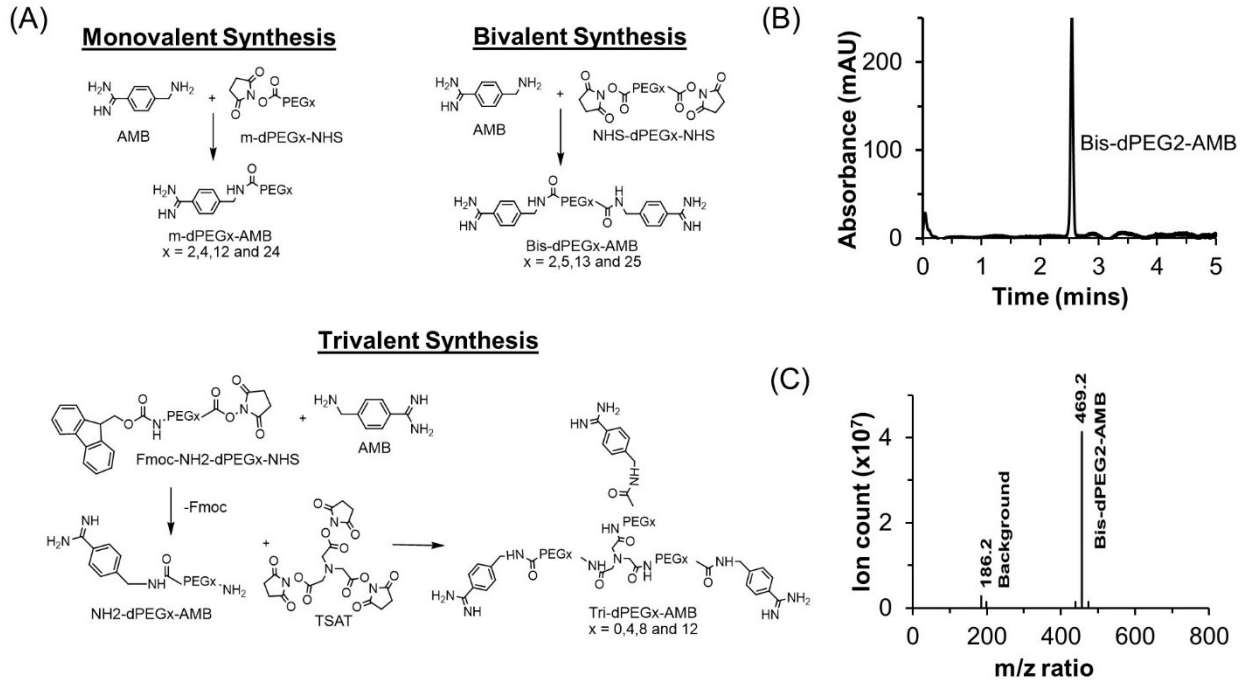

Figure 2. (A) Scheme for monovalent, bivalent and trivalent benzamidine syntheses. Exemplary data for synthesized Bis-dPEG2–AMB: B) HPLC chromatogram C) Mass spectrum.

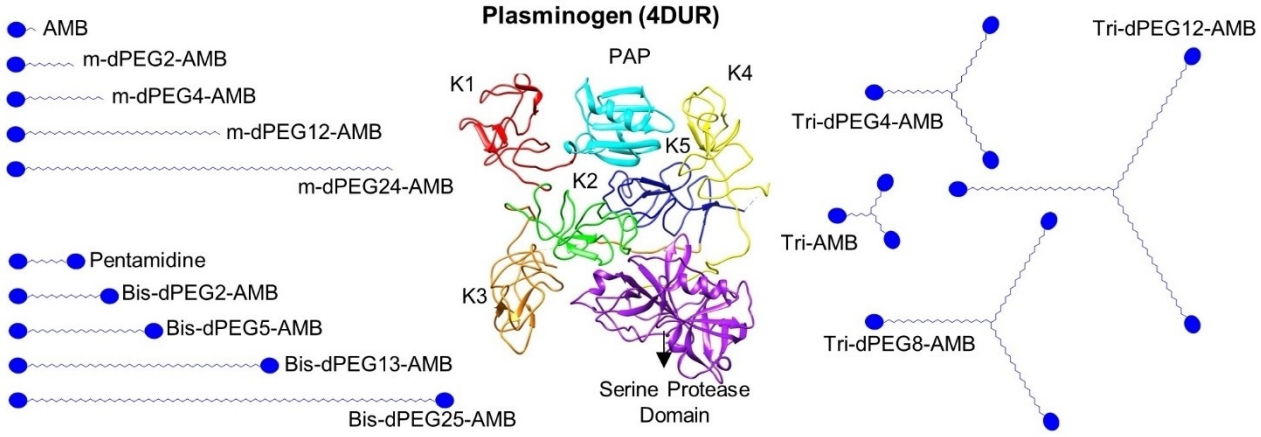

Figure 3. Monovalent (m-dPEGx–AMB), bivalent (Bis-dPEGx–AMB) and trivalent benzamidine (Tri-dPEGx–AMB) inhibitors shown to scale relative to plasminogen (PDB ID: 4DUR).

Bis-dPEG2–AMB have been included as exemplary data (Figure 4A and 4B) with additional plots provided as Figures S13–S26 in Supporting Information.

Multivalency analysis: Parameters used to determine multivalent effect, namely, relative potency (rp), and relative potency per unit (rp/n) were computed. To evaluate how strong the multivalent inhibitor is compared to the monovalent inhibitor, rp is used and is a ratio of K_i^{mono} to K_i^{multi}. K_i of monovalent AMB was used for this calculation since AMB was utilized for synthesis of all inhibitors. While rp >1 suggests that the multivalent inhibitor is stronger, it does not take into account the increased inhibitor concentration associated with multivalent inhibitor molecules. To better evaluate multivalency effects, rp/n was computed to determine the benefit of linking multiple inhibitor moieties together. A rp/n value >1 indicates that the potency of each inhibitor in the multivalent system is stronger than the monovalent inhibitor. If rp/n $=1$, it demonstrates that there is no benefit of linking inhibitors together and it is equivalent to having "n" number of monovalent inhibitors in solution. Finally, if rp/n <1, linking inhibitors together is detrimental.[1] The rp and rp/n values of all inhibitors are shown in Table 1.

All synthesized monovalent benzamidines were more potent than AMB with rp and rp/n values >1. This is potentially

Table 1. Inhibition constants (K_i), rp, rp/n values for monovalent, bivalent and trivalent inhibitors along with their separation lengths.

	Inhibitors	K_i [μM]	rp[a]	rp/n[b]	Length[c]
Monovalent	AMB	1,395 ± 165.8	–	–	1.09
(n = 1)	m-dPEG2–AMB	259.4 ± 35.9	5.4	5.4	2.13
	m-dPEG4–AMB	308.9 ± 21.5	4.5	4.5	2.91
	m-dPEG12–AMB	359.9 ± 35.2	3.9	3.9	6.19
	m-dPEG24–AMB	521.1 ± 84.9	2.7	2.7	11.03
Bivalent	Pentamidine	2.1 ± 0.8	664.3	332.1	2.37
(n = 2)	Bis-dPEG2–AMB	55.3 ± 5.3	25.2	12.6	3.34
	Bis-dPEG5–AMB	44.3 ± 5.3	31.5	15.7	4.48
	Bis-dPEG13–AMB	131.4 ± 23.8	10.6	5.3	7.64
	Bis-dPEG25–AMB	290.4 ± 95.8	4.8	2.4	12.39
Trivalent	Tri-AMB	3.9 ± 1.7	357.7	119.2	2.25
(n = 3)	Tri-PEG4–AMB	50.5 ± 14	27.6	9.2	5.77
	Tri-PEG8–AMB	130.8 ± 15.9	10.7	3.6	8.66
	Tri-PEG12–AMB	241.9 ± 34.6	5.8	1.9	11.40

[a] rp: relative potency = K_i^{AMB}/K_i^{multi}. [b] rp/n: relative potency/number of benzamidine units. [c] calculated planar separation lengths between benzamidine moieties measured end to end using ChemDraw (Version 19.0.1.)

because the change in substituent group of the aromatic ring of benzamidine alters hydrophobic interactions between benzamidine and plasmin thus, effecting inhibition.[31] This was also seen in the study carried out by Alves *et al.*, where benzamidines with different substituent groups exhibited a range of inhibition constants varying from 32 μM to 1074 μM potentially due to change in hydrophobic and charge-charge interactions.[27] All synthesized bivalent and trivalent inhibitors exhibited beneficial multivalent effects with rp/n values >1. The rp/n values for bivalent and trivalent inhibitors ranged from 2.4 to 332.1 and 1.9 to 119.2, respectively. This demonstrates that the potency of each benzamidine in these inhibitors is at least ~2-fold stronger than monovalent AMB and therefore, it is beneficial to link benzamidines together. Pentamidine, the shortest bivalent inhibitor was the strongest multivalent plasmin inhibitor with a K_i value of 2.1 ± 0.8 μM, rp of 664.3 and rp/n of much greater than 1 (332.1) comparable to the shortest trivalent inhibitor Tri-AMB of K_i 3.9 ± 1.7 μM.

When K_i values of these inhibitors were plotted against separation lengths between benzamidine moieties, it was observed that K_i increased with length indicating weaker inhibition with longer linker lengths (Figure 4C and Supporting Information Figure S27). As the chosen linker lengths are relatively short they are expected to promote statistical rebinding and not achieve other modes of multivalency such as clustering or chelation. Longer linker lengths reduced the effective concentration of the inhibitor in the vicinity of the enzyme and resulted in weaker inhibition.[32] In addition, as PEG is a flexible linker, the longer it is, the more conformational states it can occupy. This causes a higher entropic penalty for binding and more conformations that result in reduced local concentration of inhibitor molecules leading to a higher K_i value (weaker inhibition).[33] Therefore, shorter linker lengths improve inhibition by increasing the effective local concentration of inhibitor while minimizing entropic penalty.

It was also interesting to note that not only did inhibition decrease with length, but also was linearly correlated ($R^2 > 0.94$, Figure 4C). Moreover, mono-, bi-, and trivalent inhibitors had similar slopes indicating similar rate of increase in K_i (or decrease in inhibition) per increase in unit length across all inhibitors irrespective of their valencies. Also, comparing a specific separation length across valencies demonstrated that higher valency results in a smaller K_i value, or stronger inhibition (Trivalent > Bivalent > Monovalent inhibition). This is because valency is a crucial parameter that affects multivalent inhibition. Increase in valency indicates that the number of inhibitor moieties increase which in turn increases the effective local concentration of the inhibitor in the vicinity of the enzyme and therefore, promotes stronger inhibition.[33] Although, trivalent inhibitors were stronger than bivalent inhibitors, both exhibited similar K_i values and hence, higher order valencies were not tested expecting the difference to be even less substantial.

Conclusions

This study concludes that varying valency and linker length can modulate multivalent inhibition by impacting statistical rebinding. Higher valency and shorter linker lengths result in stronger inhibition. Higher valency improves inhibition by increasing the effective local concentration of benzamidine in the vicinity of plasmin and enhances statistical rebinding. Shorter linker lengths improve inhibition by increasing the effective local concentration of inhibitor and by minimizing entropic penalty. As benzamidine derivatives also inhibit other serine proteases such as trypsin and thrombin, valency and linker length of multivalent benzamidines can be optimized to inhibit desired serine protease enzyme selectively. Moreover, this strategy of modifying valency and linker length can be applied to any enzyme of interest to achieve desired inhibition.

Experimental Section

Materials: Monodisperse polyethylene glycol (PEG) linkers: m-dPEGx–NHS esters (x = 2, 4, 12 and 24), Bis-dPEGx–NHS esters (x = 2, 5, 13 and 25) and Fmoc–dPEGx–NHS esters (x = 4, 8 and 12) were purchased from Quanta BioDesign (Plain City, OH). AMB (4-

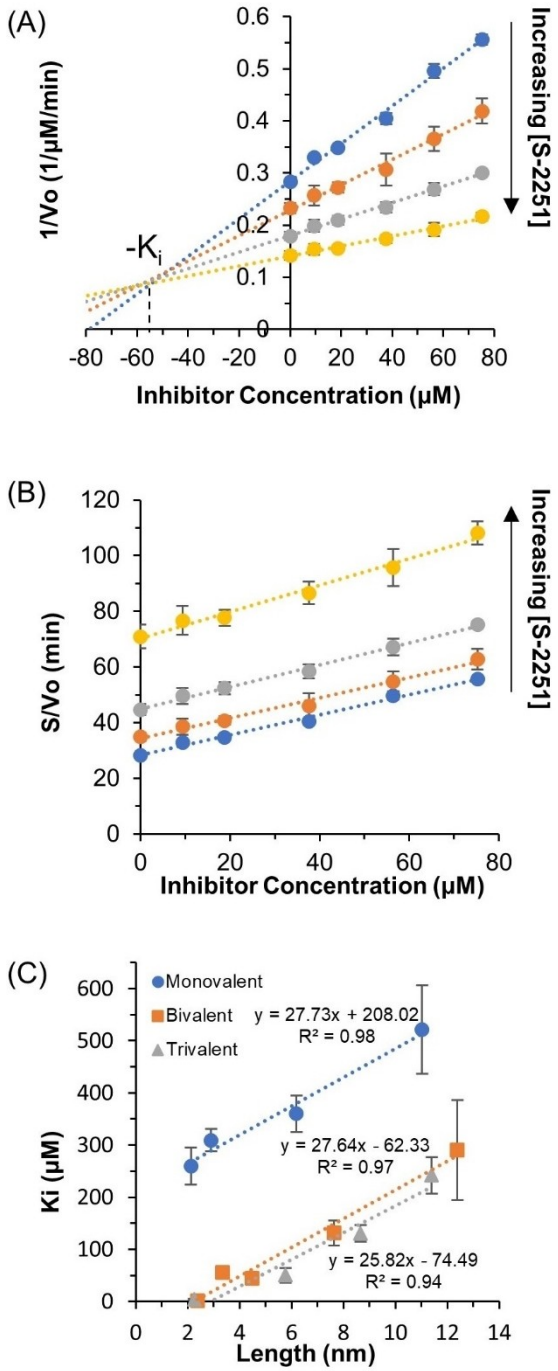

Figure 4. (A) Dixon plot for Bis-dPEG2–AMB. K_i is the magnitude of negative x intersection ($K_i = 55.3 \pm 5.3$ μM). (B) Cornish-Bowden (S/Vo vs I) plot for Bis-dPEG2–AMB. Parallel lines indicate competitive inhibition. (C) K_i vs Length for all inhibitors. Greater K_i (weaker inhibition) for longer separation lengths between benzamidines. For a specific separation length, K_i of monovalent (circles) > bivalent (squares) > trivalent (triangles) indicating trivalent exhibits strongest inhibition followed by bivalent and then monovalent.

aminomethyl benzamidine) was obtained from Aurum Pharmatech (Plano, TX) and TSAT (tris-(succinimidyl)aminotriacetate) was purchased from Thermo Fisher Scientific (Waltham, MA). Plasmin from human plasma, lyophilized was purchased from Athens Research & Technology (Athens, GA). Diapharma Chromogenix S-2251 (H–D–Val–Leu–Lys–pNA·2HCl) and Corning™ 96-Well Nonbinding Surface (NBS™) Microplates (3641) were purchased from Fisher Scientific (Waltham, MA).

Inhibitors Design and Synthesis

Synthesis Procedure for Monovalent Inhibitors: Monovalent Inhibitors (m-dPEGx–AMB; x = 2, 4, 12, and 24) were synthesized using amine reactive NHS chemistry utilizing AMB and monodisperse m-dPEGx–NHS esters (x = 2, 4, 12 and 24). The reactions were performed in 0.01 M Phosphate Buffer Saline (PBS), pH 7.4 at room temperature (RT). The synthesized inhibitors were purified using reverse phase HPLC and the masses were confirmed with mass spectrometry. Finally, analytical HPLC was performed on the purified inhibitors and the purity of the inhibitors was determined to be greater than 95%.(See Supporting Information Figures S1–S4 for additional synthesis details, ChemDraw Structures, HPLC traces and m/z values of monovalent inhibitors).

Synthesis procedure for bivalent inhibitors: Bivalent Inhibitors (Bis-dPEGx–AMB; x = 2, 5, 13, and 25) were synthesized conjugating AMB to Bis-dPEGx–NHS esters (x = 2, 5, 13 and 25) in 0.01 M PBS, pH 7.4 at RT. The synthesized inhibitors were purified using reverse phase HPLC and the masses were confirmed with mass spectrometry. The purity of these inhibitors was determined to be greater than 95% using analytical HPLC (See Supporting Information Figures S5–S8 for additional synthesis details, ChemDraw Structures, HPLC traces and m/z values of bivalent inhibitors).

Synthesis procedure for trivalent inhibitors: To synthesize trivalent benzamidine inhibitors (Tri-dPEGx–AMB; x = 0, 4, 8, and 12), AMB was first reacted with Fmoc–dPEGx–NHS esters to form Fmoc–dPEGx–AMB (x = 0, 4, 8, and 12) in DMF/PBS/TEA. Fmoc was then deprotected using ~40% Piperidine in DMF to obtain NH2–PEGx–AMB (x = 0, 4, 8, and 12) which was then purified using reverse phase HPLC. These molecules were further reacted with a trivalent core TSAT in DMF/TEA at RT to obtain Tri-dPEGx–AMB (x = 0, 4, 8, and 12). These products were purified using reverse phase HPLC and confirmed by mass spectrometry. Analytical HPLC was performed to verify the purity to be greater than 95% (See Supporting Information Figures S9–S12 for additional synthesis details, ChemDraw Structures, HPLC traces and m/z values of trivalent inhibitors).

Procedure for inhibition assays: Inhibition assays were performed on all synthesized inhibitors in addition to free AMB and Pentamidine to determine inhibition constants (K_i). For these assays, a chromogenic substrate (Chromogenix S-2251: H–D–Val–Leu–Lys–pNA·2HCl) specific for plasmin was used at a fixed concentration of plasmin (42.5 nM) over a range of substrate (100–500 μM) and inhibitor concentrations (0–1,200 μM). Initial velocities (V_o) in μM/min were calculated for each inhibitor and substrate concentration. To obtain V_o values, the slope of release of p-Nitroaniline by hydrolysis of S-2251 by plasmin was determined at 405 nm in a Corning™ 96-Well Nonbinding Surface (NBS™) Microplates (3641) using Molecular Devices SpectraMax® M5 Microplate Reader. K_i values were calculated using the V_o values obtained for each inhibitor at different inhibitor and substrate concentrations via Dixon Plot analysis using the negative x-intersection point.[34] All Dixon Plots are provided as Figures S13–S26 in Supporting Information. Cornish-Bowden graphs (S/Vo vs I) were also plotted to verify the type of inhibition, i.e., competitive, uncompetitive, non-competitive, or mixed.[35] The inhibition was determined to be purely competitive for all multivalent benzamidines as indicated by parallel lines on the Cornish-Bowden plots (Supporting Information Figures S13–S26).

Supporting Information

Additional synthesis details, ChemDraw structures, HPLC traces, m/z values, Dixon plots and Cornish-Bowden plots for all inhibitors. The Supporting Information is available free of charge via the Internet.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Abbreviations

AMB, 4-aminomethyl benzamidine; EACA, ε-aminocaproic acid; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PEG, polyethylene glycol; rp, relative potency; rp/n, relative potency per unit; TSAT, tris-(succinimidyl)aminotriacetate; TXA, tranexamic acid.

Acknowledgements

This work was supported by the Department of Emergency Medicine at the Indiana University School of Medicine. The authors thank Dr. Lifan Zeng and Chemical Genomics Core at Indiana University School of Medicine for providing access to mass spectrometry.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Benzamidine · enzymes · inhibitors · multivalency · plasmin

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Manuscript received: July 7, 2022
Revised manuscript received: September 15, 2022
Accepted manuscript online: September 16, 2022
Version of record online: October 10, 2022