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Enhanced potency of the metalloprotease inhibitor TAPI-2 by multivalent display

Aram J. Raissi^{a,†}, Frank A. Scangarello^{a,b,†}, Kaitlin R. Hulce^b, Jason K. Pontrello^{b,*}, and Suzanne Paradis^{a,*}

^aDepartment of Biology, National Center for Behavioral Genomics, and Volen Center for Complex Systems, Brandeis University, Waltham, MA 02454, United States

^bDepartment of Chemistry, Brandeis University, Waltham, MA 02454, United States

Abstract

Metalloproteases regulate a vast array of critical cellular processes such as proliferation, migration, repair, and invasion/metastasis. In so doing, metalloproteases have been shown to play key roles in the pathogenesis of multiple disorders including arteriosclerosis, arthritis, cancer metastasis, and ischemic brain injury. Therefore, much work has focused on developing metalloprotease inhibitors to provide a potential therapeutic benefit against the progression of these and other diseases. In order to produce a more potent inhibitor of metalloproteases, we synthesized multivalent displays of a metalloprotease inhibitor derived from the ring-opening metathesis polymerization (ROMP). Specifically, multivalent ligands of a broad-spectrum metalloprotease inhibitor, TAPI-2, were generated upon conjugation of the amine-bearing inhibitor with the ROMP-derived *N*-hydroxysuccinimide ester polymer. By monitoring the metalloprotease dependent cleavage of the transmembrane protein Semaphorin4D (Sema4D), we demonstrated an enhancement of inhibition by multivalent TAPI-2 compared to monovalent TAPI-2. To further optimize the potency of the multivalent inhibitor, we systematically varied the polymer length and inhibitor ligand density (mole fraction, χ). We observed that while ligand density plays a modest role in the potency of inhibition caused by the multivalent TAPI-2 display, the length of the polymer produces a much greater effect on inhibitor potency, with the shortest polymer achieving the greatest level of inhibition. These findings validate the use of multivalent display to enhance the potency of metalloprotease inhibitors and further, suggest this may be a useful approach to enhance potency of other small molecule towards their targets.

Keywords

Multivalent; Metalloprotease; Semaphorin4D; TAPI-2; Cleavage

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*Corresponding authors. Tel.: +1 7817365305 (S.P.). pontrell@brandeis.edu (J.K. Pontrello), paradis@brandeis.edu (S. Paradis).

†Authors contributed equally.

Supplementary data

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Metalloproteases are a class of enzymes that utilize an active site zinc ion to cleave peptide bonds. More than one hundred different metalloproteases have been identified: many are classified as either secreted matrix metalloproteases (MMPs) or A Disintegrin and Metalloproteases (ADAMs), the majority of which are type I transmembrane proteins.¹⁻³

Functionally, metalloproteases have been implicated in a vast array of biological processes, ranging from vascularization and organogenesis in early development to inflammation and tumor cell metastasis in mature organisms, by directly regulating the cellular signaling environment through cleavage of intracellular and membrane associated proteins.⁴⁻⁸ For example, disruption of ADAM10-mediated cleavage of the Notch extracellular domain in mice disrupts skin formation and maintenance by triggering a premature differentiation of spinous keratinocytes in the embryo and hyperproliferation of basal keratinocytes.⁹ Similarly, disruption of ADAM10 function in the developing central nervous system leads to precocious neuronal differentiation and a misformed cortex, again as a result of disrupted Notch signaling.¹⁰ Therefore, due to their wide-ranging functions in cellular processes and their role in disease, much effort has focused on discovery of metalloprotease inhibitors.

To ask if we could enhance the potency of an existing hydroxamate-based metalloprotease inhibitor, TAPI-2, we took the novel approach of conjugating the small molecule synthetic inhibitor to a multivalent display. TAPI-2 was initially isolated as an inhibitor of Tumor Necrosis Factor α (TNF- α) processing^{11,12} and has since been shown to be a broad spectrum inhibitor of both MMPs and ADAMs. We sought to determine if a multivalent display of TAPI-2 could enhance the inhibitor's potency. Multivalent display efficacy is based on the principle that altering the presentation of a molecule or ligand to its corresponding enzyme or receptor can greatly influence its binding affinity and ability to activate downstream biological processes. One mechanism by which this can occur is through increasing the local concentration of the ligand, which influences the frequency with which the ligand binds to its corresponding receptor.^{13,14}

Multivalent displays have been shown to greatly enhance the binding properties and biological activity of molecules both in vitro and in vivo.¹⁵⁻¹⁸ For example, inhibitor potency of various sugars towards enzyme classes such as glycosidases or α -mannosidases can be increased by conjugation to multivalent scaffolds compared to monovalent controls.¹⁹⁻²¹ Enhancement of potency with multivalent displays has also been observed in biological systems involving receptor binding, including multivalent fertilin β binding to its integrin receptor and multivalent displays of antigens targeting B-cell receptors.^{15,17,22,23} Interestingly, a recent report demonstrated that multivalent displays of the chlorotoxin peptide increased endocytosis of MMP-2 from the cell surface.²⁴

Synthetic scaffolds provide an efficient means to generate multivalent ligands able to systematically vary local ligand concentration as well as structural presentation of ligands to their binding partners. Synthetic multivalent ligands derived from the Ring-Opening Metathesis Polymerization (ROMP) have been used to study multivalent affects in a diverse array of biological systems, ranging from mechanisms of mammalian sperm-egg binding, to B-cell activation and bacterial chemotaxis.^{17,23,25} These ROMP-derived polymers have several advantages over other synthetic methods.^{17,26} Conditions can be optimized to

provide polymers with low polydispersity, allowing for the generation of discrete populations of various lengths, and exploration of the effect of polymer length on a particular interaction (Scheme 1). Polymers can be synthesized with a group that can be further synthetically modified, allowing for efficient appendage of a biological ligand of interest. Furthermore, the ROMP-derived polymer serves as a basis by which ligands containing an amine nucleophile can be conjugated to the polymer at different concentrations resulting in control over the ligand density in the multivalent display.

Therefore, we sought to use ROMP-derived polymers as scaffolds to display the metalloprotease inhibitor TAPI-2 multivalently, allowing us to explore if a multivalent display of this molecule could increase its potency as a metalloprotease inhibitor.

Certain members of the Semaphorin family have been shown to undergo metalloprotease-dependent shedding from the cell surface, which plays a critical role in their function. The Semaphorins are a large family of over 25 transmembrane and secreted glycoproteins that were originally identified as axon guidance molecules and are characterized by a conserved extracellular Semaphorin (Sema) domain.^{27,28} The transmembrane Semaphorin family member, Sema4D, has been shown to play important signaling roles in both immune response, synapse formation, and angiogenesis.^{29–33} Further, metalloprotease-dependent cleavage of Sema4D from the cell surface has been demonstrated to play a critical role in each of these processes.^{31–33}

We chose to monitor the metalloprotease-dependent cleavage of Sema4D in HEK 293T cells to determine if a multivalent display enhances the potency of the small molecule metalloprotease inhibitor, TAPI-2. Further, we sought to determine if altering the structural characteristics of the multivalent display, such as polymer length or ligand density, affects the potency of the multivalent TAPI-2. In so doing, we validated the use of multivalent display chemistry to enhance the potency of small molecule inhibitors, which implies that multivalent display could enhance the potency of other small molecules that might be expensive or difficult to synthesize.

Proteolytic cleavage of the extracellular domain of Sema4D has been demonstrated in platelets, neurons, and lymphocytes.^{30,32,34} We sought to determine if proteolytic cleavage of the extracellular domain of Sema4D is metalloprotease dependent in our culture system by overexpressing a recombinant form of Sema4D with a Flag epitope fused to the C-terminus (Sema4D-Flag) in HEK293T cells. Using Western blot analysis, we probed cellular homogenates with a Sema4D specific antibody that recognizes the C-terminus and observed a doublet band at approximately 150 kDa product corresponding to full length Sema4D as confirmed by probing with two independent antibodies that recognize Sema4D (Fig. 1 and data not shown). When cells were treated with the PMA (phorbol myristate acetate), to stimulate metalloprotease activity,³² we detected an additional 25 kDa product, corresponding to an intracellular C-terminal cleavage fragment (Fig. 1). In order to further confirm that proteolytic cleavage of Sema4D was metalloprotease dependent, we co-treated HEK293T cells expressing Sema4D with the activator PMA and either a broad spectrum metalloprotease inhibitor (GM6001) or a negative control (GM6001NC) which lacks the hydroxamic acid required for the inhibitor to bind the zinc active site of metalloproteases

(Fig. 1). We observe that co-treatment with GM6001 but not with GM6001NC leads to a decrease in the PMA induced Sema4D cleavage product in a concentration dependent manner (Fig. 1), confirming that Sema4D is proteolytically cleaved by metalloproteases in HEK293T cells.

Upon confirming that Sema4D cleavage is metalloprotease dependent in HEK293T cells, we next asked if we could increase metalloprotease inhibitor potency by using a multivalent display. To test our hypothesis, we used the prevalence of the Sema4D cleavage product as a monitor of inhibitor potency in our system. First, we generated a multivalent metalloprotease inhibitor using a post-polymerization modification strategy to conjugate inhibitor ligands onto a polymeric scaffold.^{17,26} To generate the scaffold, we employed the Ring-Opening Metathesis Polymerization reaction (ROMP) utilizing a ruthenium carbene catalyst (Scheme 1). By varying the monomer to initiator ratio ($M:I$), polymers of defined length (n) and discrete populations can be generated.^{17,35–37} To allow for ligand conjugation to the resulting polymer scaffolds, we polymerized monomer (**1**) containing an active *N*-hydroxysuccinimidyl (NHS) ester group to generate amine-reactive polymers (**3–6**). Because conjugation of our ROMP-generated polymer to a ligand requires a nucleophilic attack by amines, we chose to conjugate the broad spectrum metalloprotease inhibitor TAPI-2 with its free amine group making it an ideal candidate for the generation of a multivalent metalloprotease inhibitor (Supplemental Fig. 1).

Using TAPI-2 and the ROMP-derived scaffold, we synthesized a small library of multivalent TAPI-2 inhibitors of different polymer lengths and TAPI-2 ligand densities (Scheme 1, compounds **7–10**, a–c). Using a Monomer to Initiator ratio of 10:1 resulted in a polymer (**3**) of average length $n = 10$ ('10mer'). Variation of $M:I$ ratios further generated 25mer, 50mer, and 100mer polymer scaffolds (**4–6**). Ligands were conjugated at mole fractions ($\chi_{\text{TAPI-2}}$) of 0.1 or 0.5 to evaluate the effect of ligand density displayed from polymer scaffold. When conjugating a sub-stoichiometric amount of ligand to polymer scaffold, the remaining NHS ester sites were converted to neutral biological functionality by reaction with 3-amino-1,2-propanediol. For example, with a $\chi_{\text{TAPI-2}}$ of 0.1, the remaining 90% of NHS ester groups were converted into the diol. A control polymer of each length (**7–10**, c) displaying only diol was synthesized to verify the polymer scaffold as well as diol group did not affect the metalloprotease activity. Following ligand conjugation, the polymers were purified by size exclusion chromatography with water eluent. Products were recovered in moderate to excellent yields (44–89%) except the 100mers, which were recovered in lower yields (10–33%), likely due to reduced solubility observed with the longer polymer scaffold. NMR characterization by integration of the polymer alkene region (5–5.5 ppm, 2H) compared with the TAPI-2 *tert*-butyl and isobutyl methyl groups (0.6–0.9 ppm, 15H)^{38,39} allowed for the determination of mole fractions consistent with the stoichiometry used in the conjugation reaction ($\chi_{\text{TAPI-2}} = 0.1$ or 0.5). While a sharp signal at 3.3 ppm clearly indicated incorporation of the diol (3H CH_2OH and CHOH), significant overlap with surrounding signals prevented reliable integration. NMR spectra are provided as Supplemental information (Figs. S2–S10).

To determine whether our multivalent display of TAPI-2 could affect the potency of the small molecule inhibitor TAPI-2, we assayed the affect of multivalent TAPI-2 on inhibition

of Sema4D cleavage in HEK293T cells (Fig. 2). Similar to the effects of GM6001 observed earlier (Fig. 1) we found that addition of TAPI-2 as an unconjugated monovalent molecule leads to a reduction in the PMA induced cleavage product at a concentration of 500 nM (Fig. 2, Table 1). Interestingly, addition of 50 and 500 nM TAPI-2 conjugated to the 25-mer ROMP-polymer (Scheme 1-compound **8a** ($\chi_{\text{TAPI-2}}$ of 0.1)) caused a reduction in the appearance of the PMA induced Sema4D cleavage product (Fig. 2; Table 1), indicating enhanced inhibition (24–29%) of metalloprotease dependent cleavage of Sema4D by multivalent TAPI-2. Notably, we observe that the multivalent display enhanced the inhibitory efficacy of TAPI-2 even at a concentration at which monovalent TAPI-2 has little effect (50 nM; but see Figs. 3 and 4). We took advantage of the fact that our detection of Sema4D and its cleavage product by Western blotting is quantitative due to our use of the LiCor Odyssey imaging system (LI-COR Biosciences; see Supplemental methods). Therefore, we divided the intensity of signal corresponding to the Sema4D cleavage product by the intensity of signal corresponding to full length Sema4D and then normalized to the PMA only condition (see Supplemental methods).

The quantified data is presented in Figure 2B and summarized in Table 1. To provide a quantification of inhibition efficacy, we calculated ‘percent cleavage over monomer’ by subtracting the normalized cleavage product intensity of the TAPI-2 multivalent display from the normalized cleavage product intensity of its corresponding monomeric TAPI-2 control, multiplied by 100 (Table 1). Taken together, our data reveal an enhancement of inhibitor potency due to the multivalent presentation of the TAPI-2 small molecule inhibitor to the metalloprotease target.

One of the advantages of ROMP-derived polymer scaffolds is that they can be easily manipulated to create polymers of different length and ligand density. Therefore, we first asked if manipulation of the density of TAPI-2 molecules on the ROMP-derived polymer scaffold would enhance TAPI-2 dependent inhibition of Sema4D proteolytic cleavage. In order to address this, we synthesized two independent 25-mer polymer scaffolds consisting of $\chi_{\text{TAPI-2}}$ of either 0.1 or 0.5 (i.e., **8a** ($\chi_{\text{TAPI-2}}$ of 0.1) and **8b** ($\chi_{\text{TAPI-2}}$ of 0.5)). We co-treated HEK293T cells expressing Sema4D with PMA and either monovalent TAPI-2 at 50 nM or one of each of the polymers of varying TAPI-2 density at 50 nM, or their corresponding control scaffolds displaying only diol groups (i.e., **8c**) and monitored accumulation of the Sema4D cleavage product using our Western blot assay. We chose the 50 nM TAPI-2 concentration because, based on our results in Figure 2, we observed the most robust enhancement of multivalent TAPI-2 efficacy at this concentration.

However, we did observe a slight inhibitory effect of 50 nM monovalent TAPI-2 treatment (approx. 5–10%) in these experiments and the ones described in Figure 4. Nonetheless, we observed that treatment of polymers containing either $\chi_{\text{TAPI-2}}$ of 0.1 (i.e., **8a**) or $\chi_{\text{TAPI-2}}$ of 0.5 (i.e., **8b**) demonstrated an enhanced inhibitory efficacy (26% and 34%, respectively) on Sema4D cleavage compared to corresponding monovalent TAPI-2 (Fig. 3B, Table 1).

We next asked if a change in potency could be achieved by altering the length of the polymer scaffold. To address this question, we synthesized four $\chi_{\text{TAPI-2}}$ of 0.1 density multivalent inhibitors of varying lengths (10-mer, 25-mer, 50-mer and 100-mer) and tested

their effects at 50 nM on Sema4D cleavage using our Western blot assay. In contrast to the changes observed when we increased the density of TAPI-2, increasing the length of the polymer scaffold caused a robust decrease in potency of the multivalent display (Fig. 4, Table 1). The shortest multivalent TAPI-2 polymer scaffold (10-mer, **7a**) displayed a robust enhancement (47%) of inhibitory potency when compared to monovalent TAPI-2 (Fig. 4). Thus, we find that increasing polymer length inversely affects inhibitor potency, with the shortest multivalent TAPI-2 polymer scaffold producing the strongest inhibitory effect.

Our observations are consistent with other studies that report multivalent presentation of ligands results in enhanced potency of the ligand in a variety of diverse biological systems.

However, it has been frequently observed that longer polymers are more potent as they become able to bind to multiple protein targets. For example, others have demonstrated that increasing the length of the ROMP polymer scaffold enhances the ability of the antigen DNP to stimulate B-cell activation due to more effective clustering of B-cell receptors on the cell surface.¹⁷

Conversely, another study demonstrated that only ROMP polymers of a defined length enhanced inhibitory efficacy of the sperm peptide fertilin β binding to its egg receptor, while no additional benefit was gained by increased length of fertilin β -conjugated polymers.²² This result suggests that fertilin β binds a limited number of receptors on the egg surface and thus, recruitment of additional receptors does not enhance inhibitor potency.²² Our observation of increased TAPI-2 potency with multivalent display, coupled with decreased potency as polymer length increases, suggests that increased local concentration of the TAPI-2 molecule is critical for inhibitor potency. The results of our work emphasize the importance of systematic evaluation of polymer length and ligand density in the optimization of multivalent ligand potency. Further studies are required to evaluate the applicability our multivalent TAPI-2 display to enhance inhibitory efficacy of additional metalloprotease substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sema4D is proteolytically cleaved in HEK293T cells in a metalloprotease dependent manner. (A) Representative Western blot displaying full-length Sema4D (detected as a doublet band) and Sema4D cleavage product (as determined by our monoclonal Sema4D antibody targeting the C-terminus) in response to either No treatment, PMA only, or PMA and indicated concentration of the broad-spectrum metalloprotease inhibitor GM6001 (0.1, 1, and 10 μ M). As a negative control, cells were also treated with PMA and 10 μ M of GM6001 NC where indicated. Blots were probed with our monoclonal antibody targeting the C-terminus of Sema4D. Blots were re-probed for β -actin and GFP to account for any changes in total protein or transfection efficiency, respectively.

Figure 2.

Multivalent enhancement of TAPI-2 potency. (A) Representative Western blot displaying full length Sema4D and Sema4D cleavage product in response to either No treatment, PMA only, or PMA and indicated monovalent or multivalent TAPI-2 inhibitors of various concentrations and their corresponding controls. Blots were re-probed for β -actin and GFP to account for any changes in total protein or transfection efficiency, respectively. (B) Quantification of ratio of the cleavage product band intensity to the full-length band intensity (from Western blots as shown in A; $N = 3$ experiments) normalized to the ratio of the cleavage product/full length Sema4D for the PMA only treatment.

Figure 3.

Variation of TAPI-2 ligand density weakly influences multivalent inhibitor potency. (A) Representative Western blot displaying full-length Sema4D and Sema4D cleavage product (as determined by our monoclonal Sema4D antibody) in response to either No treatment, PMA only, or PMA and 50 nM of the indicated monovalent or multivalent TAPI-2 inhibitors of various densities and their corresponding controls. Blots were re-probed for β -actin and GFP to account for any changes in total protein or transfection efficiency, respectively. (B) Quantification of ratio of the cleavage product band intensity to the full-length band intensity (from Western blots as shown in A; $N = 3$ experiments) normalized to the ratio of the cleavage product/full length Sema4D for the PMA only treatment. Concentration of diol control (compound **8c**) was measured to match the total concentration of monomeric units of each multivalent TAPI-2 inhibitor (compounds **8a** and **8b**).

Figure 4. Variation of polymeric scaffold length strongly influences multivalent inhibitor potency. (A) Representative Western blot displaying full-length Sema4D and Sema4D cleavage product (as determined by our monoclonal antibody) in response to either No treatment, PMA, or PMA and indicated 50 nM of the indicated monovalent or multivalent TAPI-2 inhibitors of various lengths and their corresponding controls. Blots were re-probed for β-actin and GFP to account for any changes in total protein or transfection efficiency, respectively. (B) Quantification of ratio of the cleavage product band intensity to the full-length band intensity (from Western blot shown in A; $N = 3$ experiments) normalized to the ratio of the cleavage product/full length Sema4D for the PMA only treatment.

Scheme 1.

Synthesis of multivalent metalloprotease inhibitors. The norbornene *N*-hydroxysuccinimide (NHS) ester monomer (**1**) was polymerized by Grubbs's catalyst (**2**) by the Ring-Opening Metathesis Polymerization (ROMP) to give polymers (**3–6**) of varying average length (n) based on the stoichiometric monomer (M) to initiator (I) ratio. Conjugation of amine-bearing ligands through amide-bond formation resulted in multivalent displays of varying lengths (**7–10**) with different mole fractions of TAPI-2 small molecule metalloprotease inhibitor (a: $\chi_{\text{TAPI-2}} = 0.1$, b: $\chi_{\text{TAPI-2}} = 0.5$). Additionally, control polymers without TAPI-2 (**7–10**, c) were synthesized.

Table 1

Summary of enhanced inhibition by multivalent TAPI-2 display

	TAPI-2 compound number	Treatment condition	Normalized cleavage product intensity [*]	% Cleavage over monomer ^{**}
Figure 2	–	PMA only	1.00	
	8c	5 nM 25mer $\chi_{\text{TAPI}} = 0.0$	1.03	
	–	5 nM Monovalent TAPI-2	0.99	
	8a	5 nM 25mer $\chi_{\text{TAPI}} = 0.1$	1.02	~0
	–	50 nM Monovalent TAPI-2	1.01	
	8a	50 nM 25mer $\chi_{\text{TAPI}} = 0.1$	0.72	29
	–	500 nM Monovalent TAPI-2	0.94	
Figure 3	8a	500 nM 25mer $\chi_{\text{TAPI}} = 0.1$	0.70	24
	–	PMA only	1.00	
	–	50 nM Monovalent TAPI-2	0.90	
	8a	50 nM 25mer $\chi_{\text{TAPI}} = 0.1$	0.64	26
	8c (to match 8a)	50 nM 25mer $\chi_{\text{TAPI}} = 0.0$	1.04	
	8b	50 nM 25mer $\chi_{\text{TAPI}} = 0.5$	0.56	34
Figure 4	8c (to match 8b)	50 nM 25mer $\chi_{\text{TAPI}} = 0.0$	1.01	
	–	PMA only	1.00	
	–	50 nM Monovalent TAPI-2	0.89	
	7a	50 nM 10mer $\chi_{\text{TAPI}} = 0.1$	0.42	47
	7c	500 nM 10mer $\chi_{\text{TAPI}} = 0.0$ control	1.20	
	8a	50 nM 25mer $\chi_{\text{TAPI}} = 0.1$	0.76	13
	8c	500 nM 25mer $\chi_{\text{TAPI}} = 0.0$ control	1.19	
	9a	50 nM 50mer $\chi_{\text{TAPI}} = 0.1$	0.91	~0
	9c	500 nM 50mer $\chi_{\text{TAPI}} = 0.0$ control	1.14	
	10a	50 nM 100mer $\chi_{\text{TAPI}} = 0.1$	1.10	~0
	10c	500 nM 100mer $\chi_{\text{TAPI}} = 0.0$ control	1.01	

*=[Experimental cleavage intensity A.U./experimental full length intensity A.U.]/[PMA only cleavage product intensity A.U./PMA only full length intensity A.U.].

**=(Normalized monomer cleavage intensity – normalized polymer cleavage intensity) × 100.