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Small Molecule Probes That Perturb A Protein-protein Interface In Antithrombin

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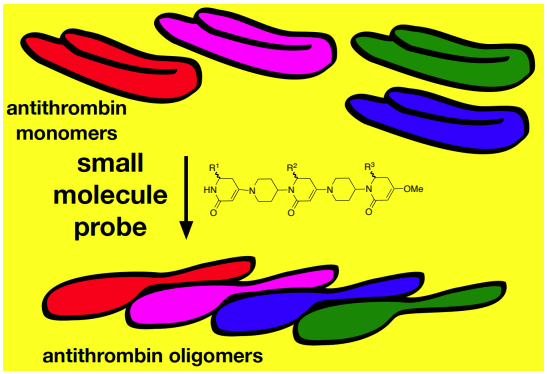
FOR GRAPHICAL ABSTRACT (please reduce to required size)



ABSTRACT:

Small molecule probes for perturbing protein-protein interactions (PPIs) *in vitro* can be useful if they cause the target proteins to undergo biomedically relevant changes to their tertiary and quaternary structures. Application of the Exploring Key Orientations (EKO) strategy (*J. Am. Chem. Soc.*, 2013, **135**, 167 – 173) to a piperidinone-piperidine chemotype **1** indicated specific derivatives were candidates to perturb a protein-protein interface in the α-antithrombin dimer; those particular derivatives of **1** were prepared and tested. In the event, most of them significantly accelerated oligomerization of monomeric α-antithrombin, which is metastable in its oligomeric state. This assertion is supported by data from gel electrophoresis (non-denaturing PAGE; throughout) and probe-induced loss of α-antithrombin's inhibitor activity in a reaction catalyzed by thrombin. Kinetics of α-antithrombin oligomerization induced by the target compounds were examined. It was found that probes with *O*-benzyl-protected serine side-chains are the most active catalysts in the series, and reasons for this, based on modeling experiments, are proposed. Overall, this study reveals one of the first examples of small molecules designed to act at a protein-protein interface relevant to oligomerization of a serpin (*ie* α-antithrombin). The relevance of this to formation of oligomeric serpin fibrils, associated with the disease states known as "serpinopathies", is discussed.

INTRODUCTION

Serpins, Serpinopathies, and α-Antithrombin

Serpins are proteins that, in their monomeric forms, function as naturally occurring serine protease inhibitors. Monomeric serpins are metastable,[1] and they revert to thermodynamically more favorable (*ca* 32 kcal/mol)[2] dimeric, then oligomeric, forms. Oligomeric serpins assemble into fibrils which are associated with a series of diseases known as "serpinopathies"[3] which encompass conditions as diverse as some neurological conditions and liver sclerosis.

α-Antithrombin is a serpin that inhibits thrombin. It played an important role in the development of understanding how serpins may form fibrils because Huntington *et al* were able to crystallize a physiologically relevant dimer that now serves as a model for the formation of oligomers (the oligomers have not yet been structurally characterized on a molecular level, presumably because they are non-homogenous involving a range of molecular masses). Figure 1 illustrates how the red and purple sheet regions of the α-antithrombin monomer (PDB: 2ANT) reorganize to form a purple cleft to accept an interface hairpin from the partner, and donate a red hairpin to it in the dimer (2ZNH).[2,4] Consequently, one of the several models for serpin oligomer formation[2,4-11] is via domain swapping to form a dimer, then repetition of this process to form oligomers.[10,11] Thus serpin-dimer formation is potentially critical in serpinopathies, and has been described as "infectious".[5,12,13]

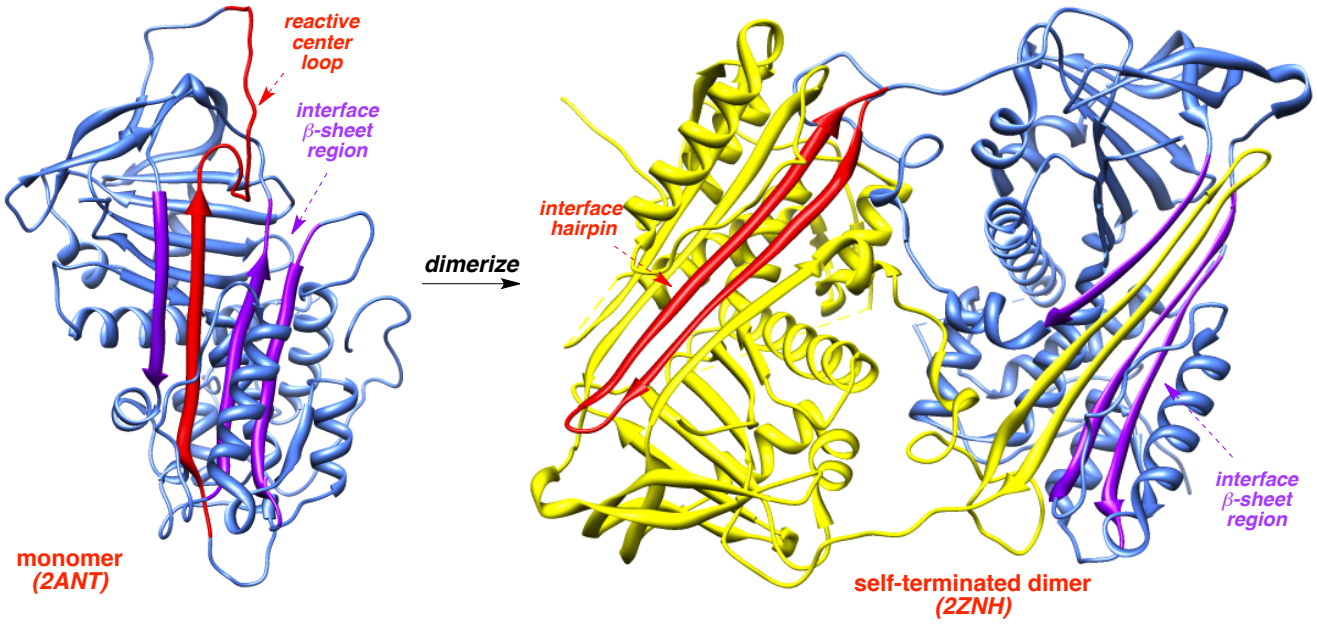
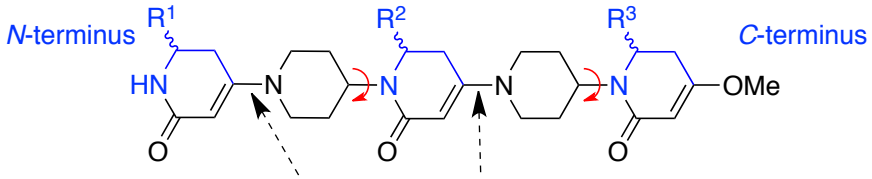


Figure 1. Structure of the α-antithrombin monomer, and the self-terminated dimer that is a putative intermediate in the oligomerization process that results in fibril formation.

Comparing Minimalist Mimic Conformations With Proteins At PPI Interfaces

EKO (Exploring Key Orientations)[14] and EKOS (Exploring Key Orientations on Secondary structures)[15] are strategies to facilitate correlations of accessible solution state conformations of certain chemotypes with protein-protein interaction (PPI) interfaces, and with secondary structures, respectively. Specifically, EKO and EKOS are designed to work with chemotypes that involve semi-rigid organic scaffolds with three amino acid side-chains. EKO is designed to identify chemotypes of this kind that perturb PPIs. It involves molecular dynamics to generate a comprehensive set of accessible conformations of these molecules, characterization of each of these conformations in terms of the side-chain orientations that they express, then data mining to match these with side-chain orientations found at PPI interfaces. The implication is that if the scaffold can present side-chains in the same orientation as an interface region involving one protein in a PPI, then it might be able to displace that protein, or at least perturb the interface.

Scaffolds **1**, as featured in this paper, are a good example of the kind of chemotype that can be processed using the EKO approach. Molecules in this series contain three β-amino acid fragments (blue) that can be made from the corresponding α-amino acid chirons, and have only two significant degrees of freedom (red arrows).



LLL-**1aaa**; *DDD*-**1asf**; *DDD*-**1as'f**; *LDD*-**1asf**; *LLD*-**1lat**; *LLD*-**1lat'**
DLL-**1lat**; *DLL*-**1lat'**; *LDD*-**1vt'a**; *LDD*-**1vva**; *DLD*-**1faf**; *DDD*-**1fff**

Synthetic protocols were recently communicated for compounds **1**.[16] Small letters in the nomenclature above refer to the corresponding amino acids, *eg* **a** indicates Me side-chain of Ala {A}, and the prime indicates a protection, *eg* **s'** is from side-chain (benzyl) protected serine. EKOS was used to compare the simulated conformations of all isomers of **1aaa** with ideal secondary structures; this indicated that stereomers of chemotypes **1** can adopt conformations that resemble all common secondary structures, with a bias towards extended sheet-turn-sheet and β-sheet conformations more than helical ones.[16]

For the work described here, EKO identified several PPI interface regions that correspond to side-chain orientations found in synthetically accessible conformations of **1**. Consequently, we set out to ascertain if the compounds **1** implicated in this EKO analysis, would in fact perturb PPIs that influence α-antithrombin oligomerization. These experiments were not performed as part of a drug discovery effort, but rather to test if EKO could be used to identify compounds that perturb PPIs in this particular test case. Specifically, the goal was to explore if the structures implicated by EKO would perturb PPIs in antithrombin and impact (positively or negatively) the oligomerization process.

RESULTS

Perturbation Of α-Antithrombin Oligomerization

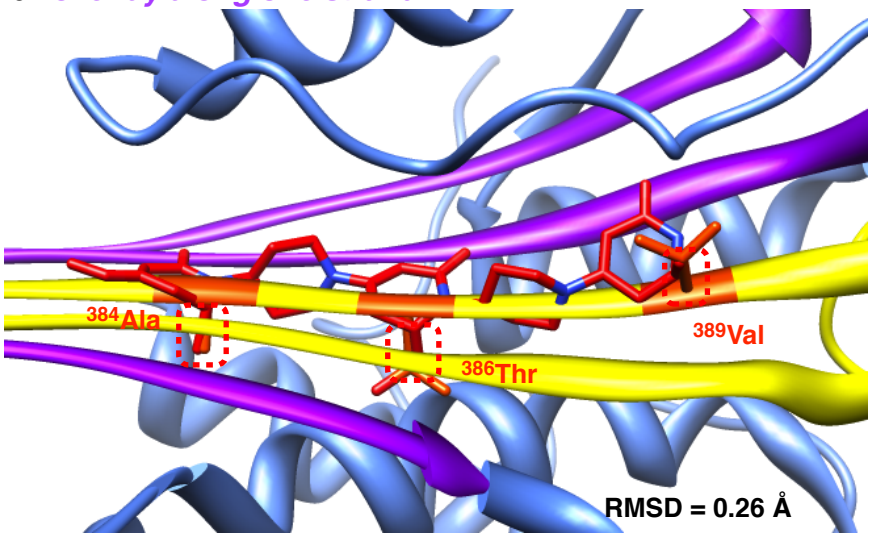
Results from EKO imply scaffold **1** might present side-chains in several ways that resemble the α-antithrombin dimer PPI interface; the four that were experimentally tested in this work are listed in Table 1, and three representative overlays are shown in Figure 2. One (RMSD 0.26 Å) involves three residues on a single strand of the β-hairpin in an *i*, *i* + *2*, *i* + *5* relationship (*i.e.* ^{384}Ala, ^{386}Thr, and ^{389}Val; Figure 2a and entry 1 in Table 1). Conversely, the scaffold spanned the *two* strands of the β-hairpin in other overlays, matching ^{373}Leu and ^{371}Ala on one strand and ^{386}Thr on the other in Figure 2b, and in Figure 2c ^{383}Ala/^{385}Ser on one strand and ^{368}Phe on the other. Another issue surrounding data mining within the EKO strategy relates to the *polarity* of the overlays. Scaffolds like **1**, being formed from amino acids, have recognizable *N*- and *C*-termini, and these can be overlaid parallel or antiparallel to the protein. In Figure 2a and b the overlay is *antiparallel* so the mimics that should be prepared are LDD-**1vta** and LLD-**1lat**. Figure 2c however, shows mimic conformers overlaid on the strands in a *parallel* fashion hence the target is DDD-**1asf** and not DDD-**1fsa**. Like entry 2, the fourth overlay referred to in Table 1 (entry 4, but not shown in Figure 2) also corresponds to the **1lat** sequence, but the stereochemistry and polarity is different.

Table 1. Conformational matches identified by EKO on interface regions in the α-antithrombin dimer structure.

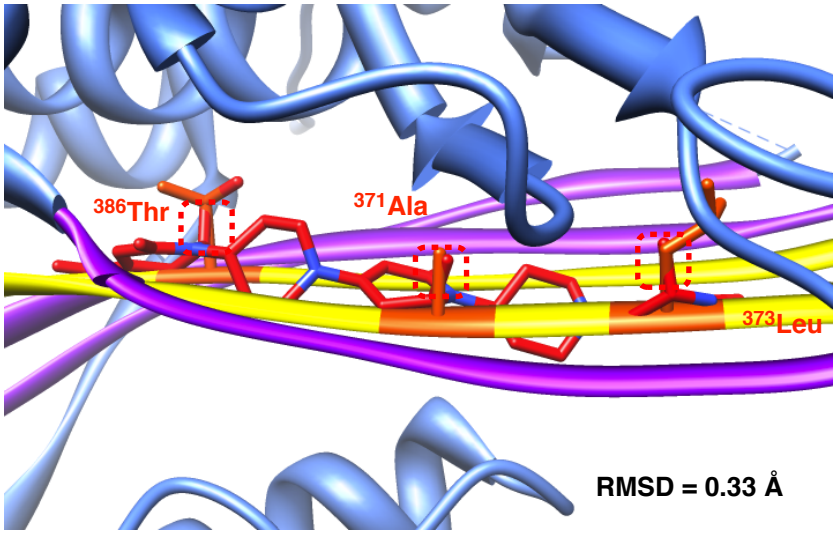
entry	isomer	RMSD(Å)	residues	polarity
1	LDD	0.26	V389-T386-A384	antiparallel

2	LLD	0.33	L373-A371-T386	antiparallel
3	DDD	0.35	A383-S385-F368	parallel
4	DLL	0.37	L373-A384-T386	parallel

a *overlay along one strand*



b *overlay across two strands*



c *overlay across two strands*

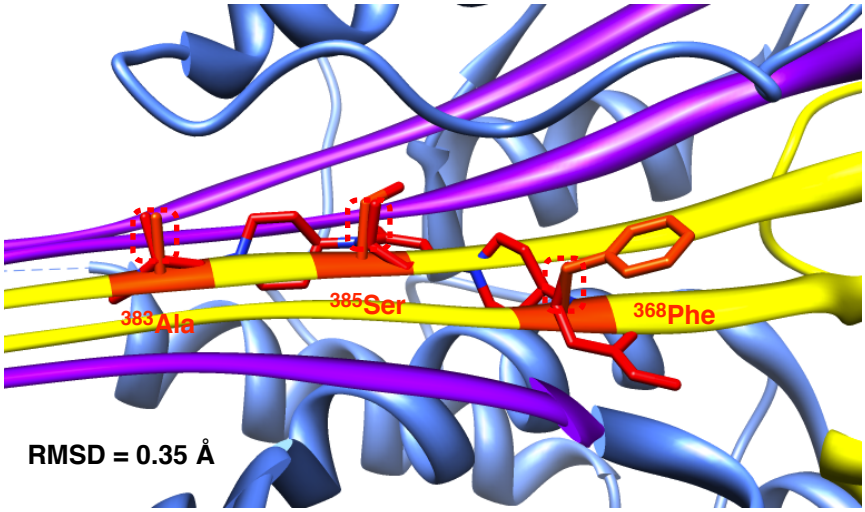


Figure 2. Conformers of scaffolds **1** can overlay side-chains on the β-hairpin structure in the α-antithrombin dimer, either: **a** on one strand; or, **b** and **c** spanning across two strands.

The compounds specified in Table 1 were prepared to test the hypothesis that EKO can implicate small molecules to disrupt PPIs in the α-antithrombin dimer,[2] but with one exception. Entry 1 of Table 1 calls for synthesis of DDL-**1vta**. However, it is considerably easier to make DDL-**1vva**, with Val replacing the Thr, because of issues with side-chain hydroxyl protection. Consequently, we elected to prepare DDL-**1vva** in place of DDL-**1vta** on the basis that the shape of the Val side-chain {$CHMe_2$} is similar to that of Thr {CH(OH)Me}.

The next step in the process was to devise assays to test perturbations of PPIs in α-antithrombin dimer. α-Antithrombin oligomerizes more readily at temperatures above ambient, and at low pH values. After some experimentation, it was shown that such oligomerizations occur at convenient rates for our purposes above 50 °C at pH 7.4. When α-antithrombin was incubated at 50 °C for 8 h at pH 7.4, it showed little sign of oligomerization as monitored via native PAGE (*i.e.* non-denaturing gel; Figure S2). Experiments were conducted to determine the effects of control or target compounds being present during incubation (Figure 3a, where target compounds are shown in red and controls or "partial controls" are shown in blue). Thus, lane 2 of Figure 3a shows that the scaffold with three methyl side-chains (LLL-**1aaa**) gave a small amount of oligomerization; observation of oligomerization in this case was unsurprising because all the EKO-implicated targets in Table 1 have a methyl side-chain and share the same scaffold, so LLL-**1aaa** is too similar to be a true negative control. However, tests with indigo, a compound that is known to bind many proteins non-specifically,[17] showed no evidence that it catalyzed oligomerization (see Figure S3).

Lanes **3**, **5** – **7** in Figure 3a showed that the four compounds targeted (DDD-**1asf**, LLD-**1lat**, DLL-**1lat** and LDD-**1vva**, in red) imparted significantly more oligomerization than LLL-**1aaa**.

Lane 4 shows data for another interesting "partial control", LDD-**1asf**. EKO did *not* indicate that conformers of this compound would match the featured PPI interface, but it might be expected to do so to some degree since it is an epimer of ones that EKO did implicate (DDD-**1asf**, lane **3**). In the event, both LDD-**1asf** and DDD-**1asf** do mediate oligomerization, but the target compound DDD-**1asf** did it more effectively than the partial control LDD-**1asf**. Finally, the gel in Figure 3a shows that all the conditions give oligomers bias towards the lower molecular mass range.

Figure 3b shows a gel that compares *benzyl-protected* forms of the targeted compounds with some similarly hydrophobic derivatives of the same scaffold **1**, but which have side-chains and/or stereochemistries that were not implicated by EKO. Lane 2 shows DDD-**1as'f**, the benzyl-protected derivative of the most active compound in Figure 3a, also catalyzed the oligomerization process. The "partial control" DDL-**1t'al** in lane 3 has two amino acids interchanged relative to the protected, targeted compound LLD-**1lat'**, and a different stereochemistry; the protected targeted compound accelerated the oligomerization most. Moreover, another protected, targeted compound having the same sequence of amino acids, DLL-**1lat'** in lane 5, also gave more acceleration than the control with two side-chains switched, DDL-**1t'al**. Finally, LDD-**1vt'a** is the only protected, target compound that did not markedly accelerate the oligomerization process. The hydrophobic compounds DLD-**1faf** and DDD-**1fff,** having the same scaffold but no side-chain correspondence, also did not promote oligomer formation significantly.

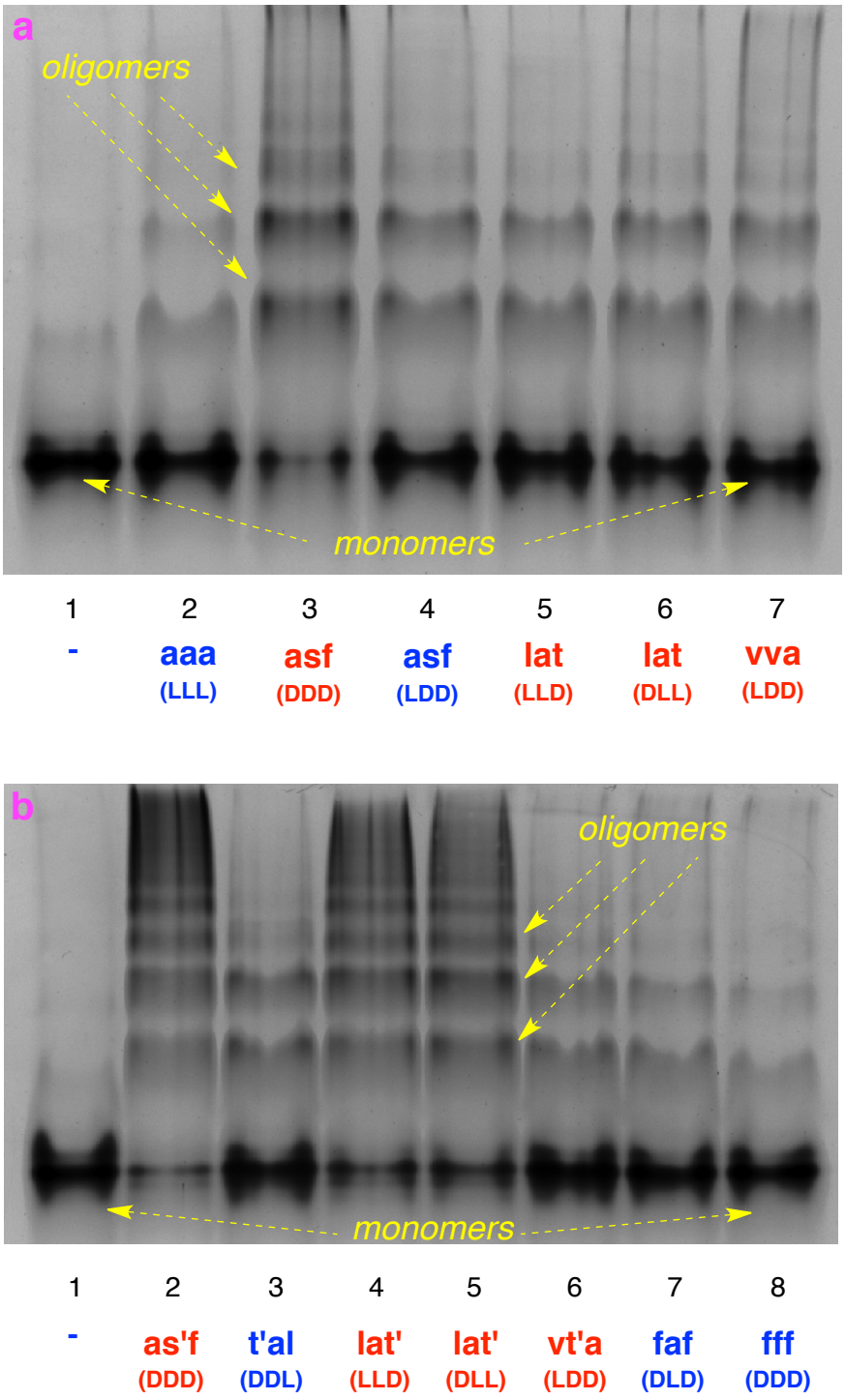
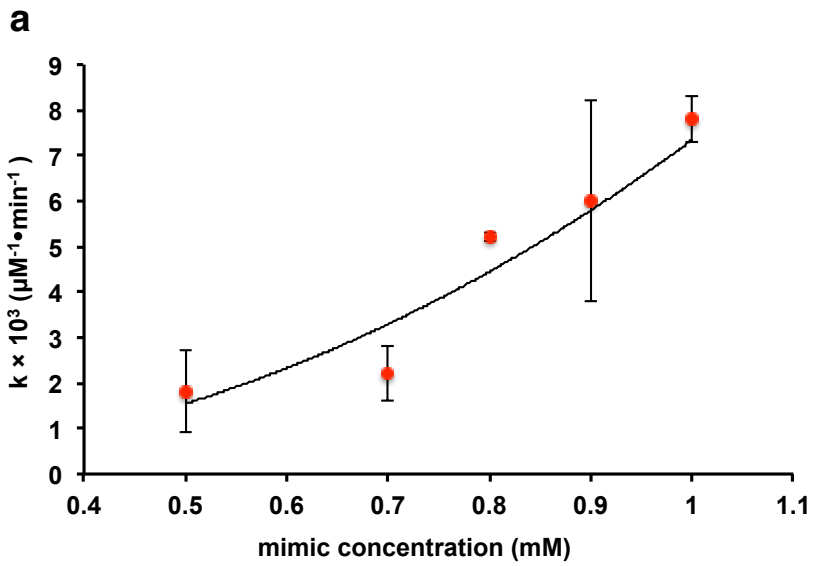


Figure 3. Throughout, controls are delineated in blue, and assays of target compounds are shown in red. After 1 h at 50 °C in pH 7.4 at 0.25 mg / mL concentration, α-antithrombin shows the following. **a** (Lane): 1 little or no oligomerization on its own; and, 2 only slightly more in the presence of the scaffold LLL-**1aaa** (only methyl side-chains). However, lane 3 shows a target compound implicated in EKO, DDD-**1asf** catalyzes formation of significantly more oligomers, particularly the ones having lower molecular mass. Lane 4 shows oligomer formation is slightly suppressed for LDD-**1asf** relative to DDD-**1asf** (corresponding to one inverted stereocenter). Lanes 5 – 7 show the indicated target compounds implicated by EKO also induce oligomerization, though less than DDD-**1asf** under the same conditions. **b** A similar

comparison for some *benzyl-protected* target compounds (red) and protected or otherwise hydrophobic controls (blue). 200 fold of indicated compounds were used in all cases.

All the PAGE data presented above are based on qualitative silver-stains. Kinetic data from the band intensities were determined to quantitate and compare effects of the compounds.[18,19] Figure 4a shows an illustrative data correlation of rate constants for oligomerization of α-antithrombin with the concentration of mimic DDD-**1as'f**. In Figure 4b, rate constants for the same oligomerization but in the presence of five featured mimics are compared; DDD-**1as'f** was the best catalyst. Kinetics was not performed on all the *unprotected* mimics shown in Table 1 because it was apparent that the protected forms tend to be better catalysts.



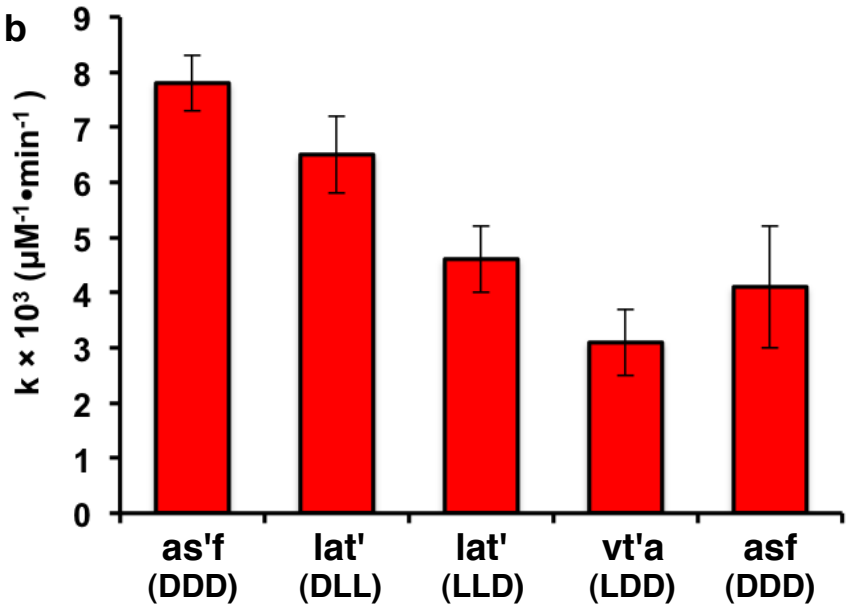
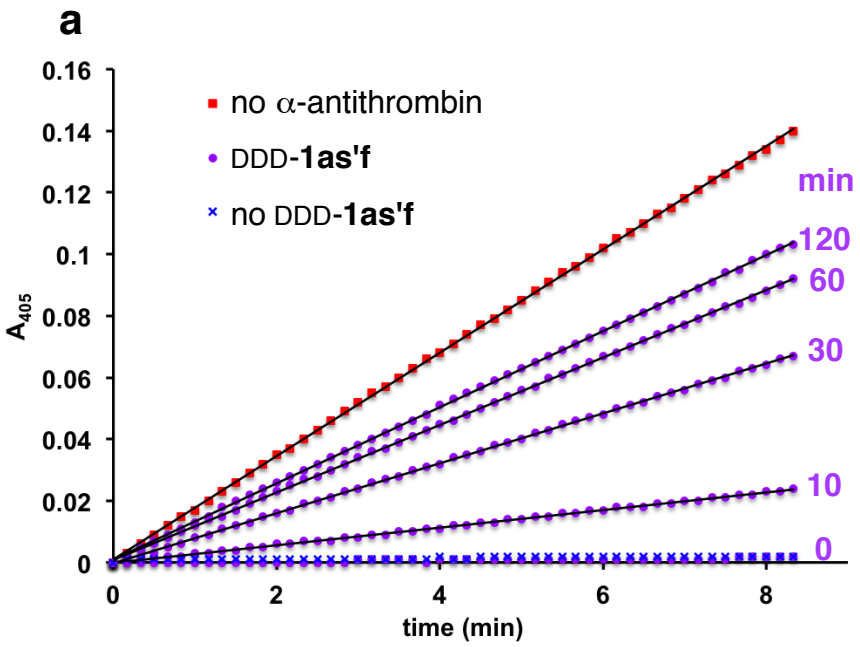
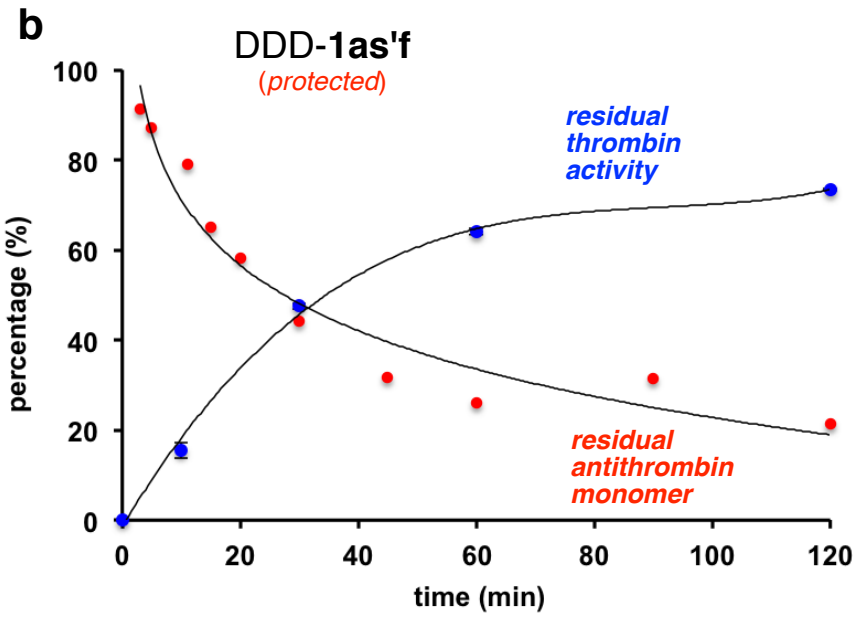


Figure 4. Kinetics of α-antithrombin oligomerization induced by: **a** DDD-**1as'f**; and, **b** benzyl-protected forms of the four featured mimics, and the best unprotected compound, DDD-**1asf** (all at 200x the protein concentration).

Another way to assay these compounds was to test antithrombin samples after oligomerization for their residual activity as thrombin inhibitors. Thus, inhibition of thrombin by α-antithrombin (optionally, after putative oligomerization mediated by the small molecule probes **1**) was used to assay the featured compounds. Thus, the probes were incubated with α-antithrombin (time variable), and aliquots of this solution were used to inhibit thrombin-mediated hydrolysis of a peptide containing 4-nitroaniline (Spectrozyme® TH, from American Diagnostica); this type of assay is a standard test for thrombin activity (performed in the presence of heparin).[20,21] In the event, thrombin was most active when inhibited by samples of α-antithrombin that had been incubated with DDD-**1as'f** (Figure 5a). This observation is consistent with the assertion made above, that DDD-**1as'f** promotes α-antithrombin *deactivation* by oligomerization well. When α-antithrombin deactivation in this assay was compared for DDD-**1as'f** and DDD-**1asf**, the benzyl-protected compound induced significantly more deactivation.


a
no α-antithrombin
DDD-1as'f
no DDD-1as'f
min
120
60
30
10
0
A405
time (min)


b
DDD-1as'f
(protected)
residual thrombin activity
residual antithrombin monomer
percentage (%)
time (min)

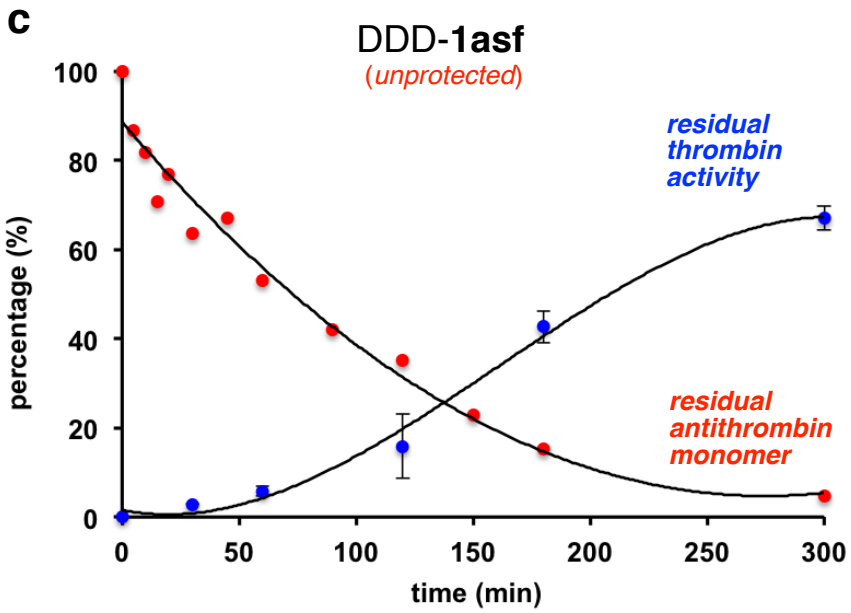


Figure 5. **a** Absorption of 4-nitroaniline formed by thrombin, and inhibited by α-antithrombin that was previously incubated with DDD-**1as'f** for the times shown. Correlation of residual thrombin activities with α-antithrombin monomer concentrations are shown in: **b** for DDD-**1as'f**; and, **c** for DDD-**1asf**.

A subtle difference in the data depicted in Figure 5 is that 50 % residual α-antithrombin monomer in 5b (for DDD-**1as'f**) corresponds to *high* thrombin activity, but in 5c (for DDD-**1asf**) 50 % residual α-antithrombin monomer corresponds to *low* thrombin activity; that observation appears to be counterintuitive. However, recall from Figure 3 the protected probe DDD-**1as'f** favors formation of *longer* α-antithrombin oligomers than the deprotected form, DDD-**1asf**. In general, formation of oligomers involves burying the free "reactive center loop" that complexes to thrombin to give inhibition in a protein cavity. α-Antithrombin oligomers have a free reactive center loop at one end, that may *still* inactivate thrombin even though the protein is oligomerized. In short oligomers, the proportion of free reactive center loops available to inactivate thrombin is *higher* than in long oligomers. Thus data in Figures 5b and c can be reconciled by accounting for the proportion of uncomplexed reactive center loops in the oligomeric products.

Alterations of protein tertiary and quaternary structures are often followed using circular dichroism (CD) and isothermal calorimetry (ITC). In this study, addition of the compounds to α-antithrombin under conditions that were expected to cause oligomerization resulted in changed CD spectra, as anticipated.[13] However, the data was hard to interpret beyond this crude observation (Figure S7). This is unsurprising because transformation of an α-antithrombin tertiary structure to another similar one in the dimeric and oligomeric forms involves only small changes in elipticity. When α-antithrombin (at 20 μM) was mixed with the optimal probe, DDD-**1as'f**, under conditions up to a 1:1 ratio in an ITC bomb, the enthalpy change could not be detected. This also is not surprising because the binding of the probe to the protein may not liberate sufficient enthalpy to detect in ITC under these conditions. Overall, these experiments are hard because they are not simple binding events, but instead the molecule acts as a catalyst to induce different oligomerization states.

Whereas CD and ITC are apparently inappropriate to follow induced α-antithrombin oligomerization, electron microscopy enabled direct visualization of the process. Negatively stained untreated antithrombin molecules revealed a range of projections and a representative area is depicted in Figure 6 (inset). In the most frequent projection, the molecules assume a roughly elliptical shape. Molecular mass can be determined from the dimensions of these particles, if a 3D shape can be inferred. Assuming a prolate ellipsoid as the overall 3D shape and a partial specific volume (υ) of 0.74 ml/g[22,23] the molecular mass (m) can be calculated according to the formula m (Da) = volume of the protein (ml) x Avogadro's number x 1/υ.[23,24] Using this formula together with the long and short half axes determined as 3.1 ± 0.3 nm and 2.3 ± 0.3 nm, respectively, one arrives at a molecular mass of 56 kDa. This finding suggests that the imaged untreated particle constitute a monomeric antithrombin population as the published molecular mass for antithrombin is 58 kDa.[2] The dramatic change in appearance upon treating antithrombin with the mimic DDD-**1as'f** is readily apparent in Figure 6 which is

characterized by a large population of higher order oligomer. α-Antithrombin oligomerized by treatment with the optimal probe (DDD-**1as'f** at 50 °C) appears as small fibrils as shown in Figure 6.

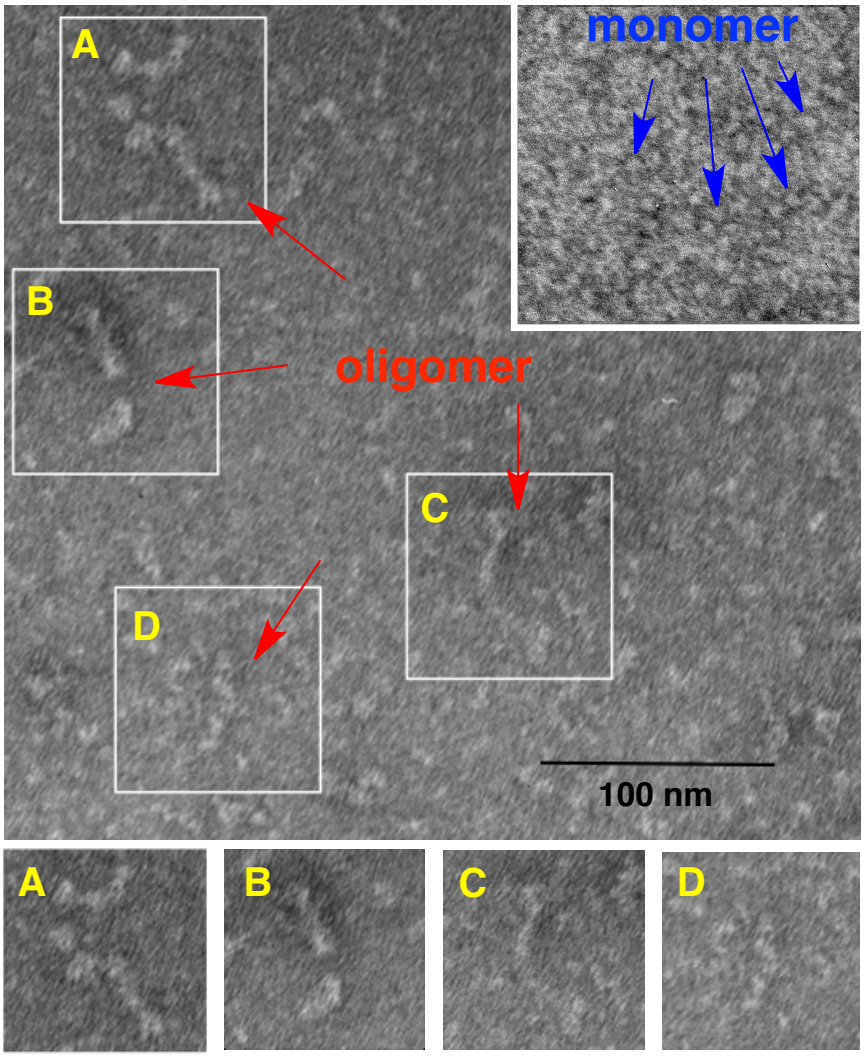


Figure 6. Electron micrographs of negatively stained α-antithrombin monomers prior to any polymerization conditions (inset); and incipient α-antithrombin fibrils induced by the action of DDD-**1as'f** at 50 °C for 1 h. Expansions A – C highlight regions of interest where the oligomers can be directly observed.

Finally, the featured compounds were designed for perturbation of the dimer intermediate in Figure 1, but the evidence outlined above would also be consistent with the compounds acting via perturbation of the monomer. This motivated us to consider how the monomer might fare in the EKO analysis, but there was one critical problem. The algorithm that EKO uses is

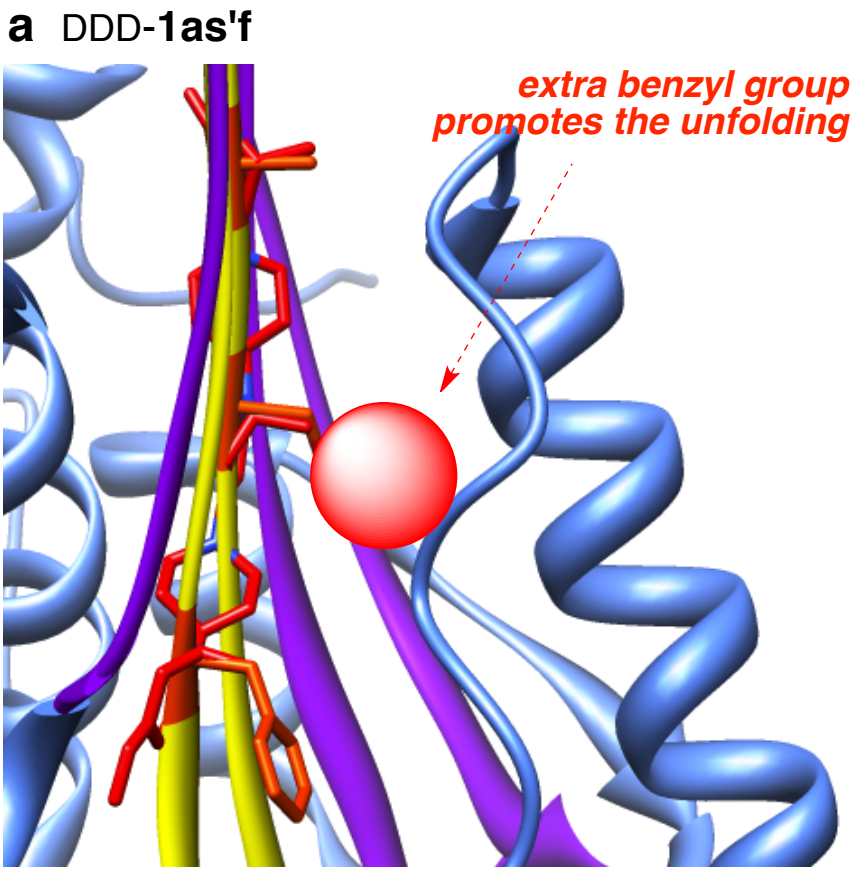
specifically designed for PPIs involving more than one chain, so it will not "pick up" hits on the antithrombin *monomer* interface. Consequently, to answer this question, we tricked the algorithm by breaking the monomer PDB so that it appears to be a PPI involving two chains. When the EKO process was applied to that *pseudo*-dimer, hits were found on the pivotal β-sheet region corresponding to in the chain swapped dimer. The results of these experiments are described fully in the supporting information.

CONCLUSIONS

Application of the EKO strategy to chemotypes **1** led us to hypothesize that selected probes in this series would perturb PPIs in α-antithrombin. In the event, four target compounds, and four benzyl-protected precursors, were tested, and all catalyzed oligomerization of α-antithrombin. This conclusion is supported by observation of oligomers on gels, by monitoring residual α-antithrombin inhibition activity in thrombin assays, and via direct observation using EM. These findings are consistent with the original hypothesis that the targeted probes perturb PPIs in α-antithrombin.

An interesting outcome of this study is that benzyl-protected forms of the target compounds proved to be better oligomerization catalysts than the non-protected compounds. This does not appear to be a non-specific hydrophobic effect because true negative controls (*eg* indigo), and closely related compounds with conformational states that were not marked by EKO as being appropriate, did not catalyze the oligomerization to the same extent. Figure 7 overlays based on EKO for two benzyl-protected target compounds (one protein removed and only protein-binding partner remains). To explain why DDD-**1as'f** is more active than the deprotected form we propose that the side-chain benzyl (shown as a red sphere in 7a) precludes the dimer protein conformation via a clash with the strand-helix motif shown on the right of that graphic. In support of this, literature observations indicate disruption of that region

may promote unfolding in the oligomerization process (based on α-antithrombin mutagenesis experiments).[25] Figure 7b suggests the binding of LLD-**1lat'** may be favored by placing the side-chain benzyl in a hydrophobic pocket.



b LLD-**1lat'**

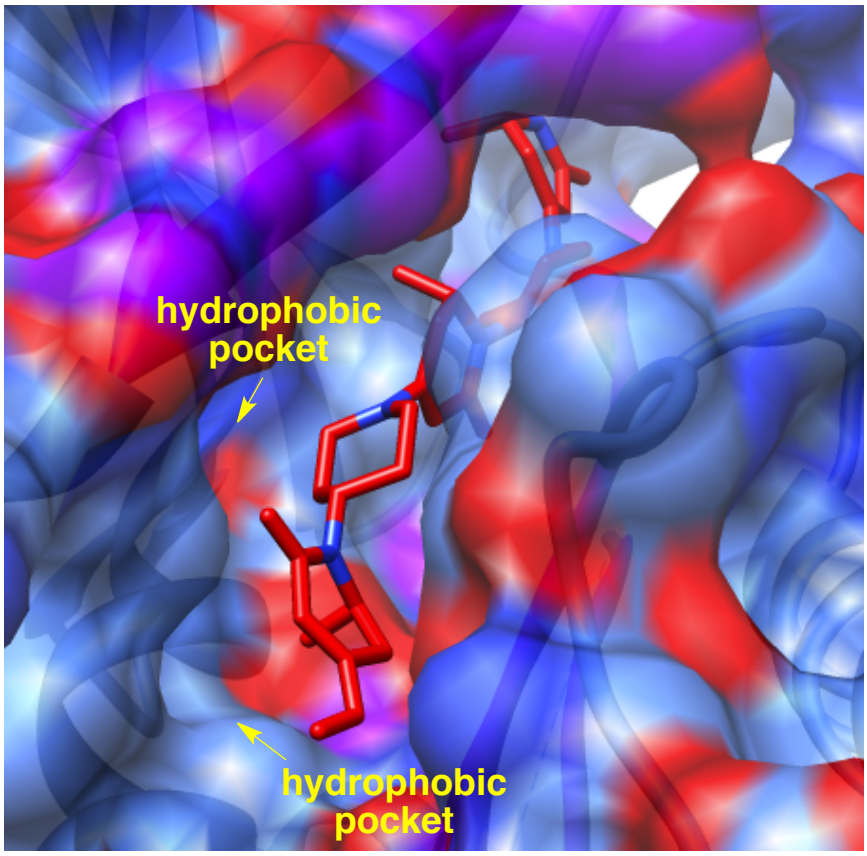


Figure 7. Proposed binding modes based on EKO analyses for: **a** DDD-**1as'f**; and, **b** LLD-**1lat'**.

Sheet and strand mimics have been reported in the literature,[26-31] but, to the best of our knowledge, no small molecules have been reported to perturb the oligomerization of α-antithrombin. One report described small molecules that perturb oligomerization of *another* serpin, a mutant of α_1-antitrypsin or "Z α_1-antitrypsin",[10] but this was discovered via virtual screening of molecules to fit in a cavity; molecules docking with that site appear to cause an allosteric interaction.[32] However, corresponding cavity is not present at the α-antithrombin PPI interface,[32] so exactly the same type of allosteric interaction is impossible for this protein. Unfortunately, elucidation of the protein regions where the small molecules impact would be hard due to the transient nature of the binding, and the heterogeneous mixture of proteins

formed in α-antithrombin oligomerization; consequently, catalysis of oligomerization via docking of the probes with an allosteric site not at the PPI interface cannot be ruled out at this stage. However, perturbation of serpin oligomerization via compounds that act at the PPI interface is possible since peptides based on this region have that effect,[33] including ones that impact Z α_1-antitrypsin[34,35] or α-antithrombin.[36,37]

The EKO strategy applied in this study led to compounds that promoted the oligomerization of α-antithrombin. EKO has no provision to determine what the biochemical effects of disrupting a particular PPI may be; in the event the perturbation here promoted α-antithrombin oligomerization.

ASSOCIATED CONTENT

Supporting Information

Procedures and other data for the EKO analyses, non-denaturing PAGE experiments, thrombin activity assay conditions, CD studies, and details for the electron microscopy. This material is available free of charge via the internet at http://pubs.acs.org.

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Notes

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