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Title of thesis: *"Effectiveness of small molecules in depolymerisation of Serine protease inhibitors"*

Serpins (*Ser*ine *P*rotease *In*hibitors) are the largest and most widely distributed family of proteinase inhibitors whose members share a common structure and mechanism of function. All members of this superfamily are biomedically relevant proteins with the members as diverse as α1-antitrypsin, α1-antichymotrypsin, C1 inhibitor, antithrombin and plasminogen activator inhibitor-1, which have key regulatory functions in the tightly regulated pathways of inflammation, complement, coagulation, and fibrinolysis respectively. The present work deals with understanding the mechanism of polymerisation of antithrombin and strategies to inhibit it. Antithrombin is the most important endogenous anticoagulant, since its heterozygous deficiency is associated with thrombotic risk while homozygous deficiency is fatal. In our study we have tried to hinder antithrombin polymerisation using small molecules. We aimed to screen out small molecules from the methylamines, sugars/ polyols and amino acids. We investigated the capacity for antithrombin polymerisation to be modulated by the presence of these small molecules in order to acquire information that may be relevant to the processes that occur *in vivo,* as well as pointing the way for the rational design of therapeutics in thrombosis as well as other serpinopathies.

We first performed an in silico screening using Autodock tools and found some molecules that bind antithrombin with high affinity. Next we did the screening by polymerisation experiments and found some lead molecules (sorbitol, mannose, trehalose, serine and TMAO) that show reduction in heat induced polymerisation of antithrombin along with concurrent increase in its activity. Nature of these polymers studied by employing transmission electron microscopy showed that antithrombin polymers have a characteristic bead like shape as observed previously for other serpins. The polymers of antithrombin reduced in size and number after incubation with small molecules. In order to see whether these molecules are affecting the normal inhibitory function and conformation of antithrombin, kinetic assays for thrombin inhibition by antithrombin were done to calculate the progressive thrombin inhibition (k_{ass}) rates. The data indicates that at polymer reducing concentration the molecules doesn't compromise the inhibitory activity of antithrombin. To evaluate protein stability, temperature induced unfolding was monitored by circular dichroism and we observed an increased *Tm* for all the small molecule treated antithrombin as compared to the native, this increase was due to an increase in the secondary structure and overall change in the tertiary structure. Secondary and tertiary structure of antithrombin was also found to be altered in the presence of lead

molecules due to an increased alpha helical content, increased hydrophilicity and increased tryptophan shielding. Activated partial thromboplastin time (aPTT), Thromboplastin Time (TT) and Prothrombin Time (PT) assays for overall effect on the rate of coagulation were performed in the presence of these small molecules. The results for the coagulation assays indicate a minor effect on the rate of coagulation on aPTT and PT assays in the presence of all small molecules. However, thrombin time showed around 3 fold increase in rate of coagulation for trehalose indicating that trehalose is implicated in the conversion of fibrin from fibrinogen by thrombin. A reduced polymer formation will allow more molecule of antithrombin to be secreted in blood and a delayed coagulation will compensate for reduced amount of antithrombin. Also a trehalose type of structural scaffold can be modified or used to build analogues that has a potential to play such dual roles and also for minimising the effective depolymerisation concentration.

We next analysed antithrombin conformations that predominate in the cytosolic soup i.e., the native state, denatured/folded-unfolded states and polymeric states. Effect of trehalose on antithrombin folding was determined after denaturing it with GdnHCl and we observed that trehalose delays formation of intermediate as compared to native. Polymerisation was also followed by a temperature dependent study and the results show that a polymerisation intermediate type partially unfolded state at around 55°C can be modulated during polymerisation whose equilibrium is shifted towards native state which decreases polymerisation. This partially unfolded state is shown to resist the transition to polymerisation through reduction in the overall hydrophobic core. Bis-ANS binding experiments with antithrombin folding intermediate also showed considerable reduction in the exposure of the hydrophobic surface in the presence of trehalose which was concurrent with an increased alpha helical content. TEM analysis also pointed that trehalose is involved in shielding antithrombin limiting the rate of polymerisation. It seems trehalose act by inhibiting the initial stage of conformational change and also the dimerization step which forces the unstable protein to fold properly by increasing the conformational stability. The results of this study show the small molecule based modulation of hydrophobic core as one of the major causes of polymer retardation.

In conclusion the study affirms the following: (a) among the lead small molecules, sugar molecules do not perturb the overall activity of antithrombin against coagulation protease of the native state and under the polymerisation condition. However they do change the secondary structure domain and hydrophobic profile of the native, intermediate and partially unfolded state. (b) Preferential hydration and large cavity binding in combination may be shifting the polymerisation equilibrium to native state. (c) Small sugars especially trehalose can be used as initial scaffold for designing molecules that hinder polymerisation but also maintain appropriate inhibitory and cofactor binding abilities in antithrombin and in other serpins.