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Title of thesis: **Preventing serine protease inhibitors polymerization using chemical chaperones: understanding the mechanism of polymerization.**

ABSTRACT

The serpins (SERine Proteinase INhibitors) are structurally similar but functionally diverse proteins that fold into a conserved structure and employ a unique suicide substrate-like inhibitory mechanism. Members of the serpin superfamily are predominantly proteinase inhibitors found in a wide range of species, including plants, viruses and humans. Serpin polymerization is a significant problem and devising a cure has been cumbersome owing to the complex mechanism of inhibition, metastable nature, cofactor binding ability and large scale conformational change of serpins. Antithrombin is used as a serpin model to understand how polymerization defect can be corrected using knowledge based strategies. Antithrombin is the most important endogenous anticoagulant, since its heterozygous deficiency is associated with thrombotic risk while homozygous deficiency is fatal.

The aim of this thesis is to know the molecular mechanism of formation of intermolecular linkages that cause antithrombin polymerization and identifies effective chemical chaperones that can reduce the hyper-coagulation indirectly by enhancement of antiprotease activity of antithrombin along with inhibiting the polymerization process. Our work utilizes chemical chaperone as chemical tools to modulate hypercoagulation, protein folding transitions and inhibition of antithrombin polymer formation along with understanding the mechanism of antithrombin based polymerization.

Towards that end, we have used large scale screening of chemical chaperones to identify compounds having potential to bind antithrombin and reduce hyper-coagulation with inhibiting polymerization. We used prothrombin time, activated partial thromboplastin time and thrombin time blood coagulation assays and native-PAGE screening and identified amino acid based (proline and GABA) and carbohydrate based (arabitol, erythritol and sucrose) chemical chaperones that are effective in delaying coagulation and inhibiting polymer formation. From *in-silico* studies (AutoDock Vina, CASTp, LIGPLOT and PEARLS) we show that these compounds also have good binding affinity and are capable to bind in large cavities in antithrombin. At polymer reducing concentrations they are also effective in maintaining antithrombin inhibitory activity and increase half-life during polymer transition experiment.

Lead compounds delay overall coagulation rates by mainly affecting the thrombin time which indicates the involvement of factor Xa and thrombin dependent regulation. Native antithrombin inhibitory activity of coagulation factor IIa (thrombin) and factor Xa indicates that at polymer reducing concentrations inhibitory activity (K_{ass}) of native antithrombin is not compromised.

However, there is an increase in stoichiometry of inhibition, indicating a delay in rate of loop insertion.

There is insignificant change on secondary structure. Bis-ANS fluorescence experiment indicated that hydrophobic patches of antithrombin are exposed in the presence of these molecules. Thermal denaturation of antithrombin at 222 nm by circular dichroism showed that there is significant increase in T_m.

Guanidine hydrochloride induced unfolding of native conformation of antithrombin in the absence and presence of lead compounds at their specific concentrations were assessed by observing the unfolding transition curves by intrinsic and extrinsic fluorescence and circular dichroism studies. Results indicate that unfolding of native antithrombin is characterized by three states (N-I-D). However, in the presence of lead compounds with exception of proline unfolding is characterized by four states (N-I_1-I_2-D).

We performed kinetic measurement of antithrombin polymerization followed by bis-ANS fluorescence. Results suggest that in the absence of lead compounds, generation of polymer occurred in two different phases. First phase is characterized by burst of bis-ANS fluorescence, suggest that formation of polymergenic intermediate, represent opening of A β-sheet (having property to accept RCL of other monomer). Second phase is characterized by decrease in bis-ANS fluorescence which suggests the polymer formation; represent insertion of RCL of one monomer into the β–sheet of other, results in expulsion of bound bis-ANS leads to decrease in intensity. When we did same experiment in the presence of lead compounds results showed reduction of initial rate and decrease in fluorescence intensity. Which conclude that opening of β-sheet during the process of polymerization is an important initial step for polymer formation and chemical chaperones prevent polymerization by inhibiting this initial β-sheet opening.

Circular dichroism study shows that sucrose inhibits polymer formation by maintaining the secondary structure probably by influencing the polymergenic intermediate to fold like native antithrombin. Further using dynamic light scattering technique, we have demonstrated time dependent changes in size of protein during polymer transition temperature in the absence and presence of sucrose. We observed that in the absence of sucrose there is continuous increase in hydrodynamic radii of protein. However in presence, change in hydrodynamic radii is insignificant. Suggesting that the preventive effect of sucrose on the protein aggregation process arises because it inhibits the initial changes in the native conformation (i.e. opening of β-sheet), which is prerequisite of polymer formation during the process of polymerization.

Overall proline, GABA, arabitol, erythritol and especially sucrose have efficiency to prolong blood hyper-coagulation and to inhibit antithrombin polymerization in their presence at specific concentrations. This type of dual role is a more appropriate therapeutic strategy to reduce both the rate of antithrombin polymerization and hyper-coagulation in patients with polymer forming variant of antithrombin that leads to thrombosis.