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Development of selective activity-based probes to study serine proteases involved in the coagulation cascade

Hemostasis (blood coagulation) is a highly regulated process triggered by damage to the endothelial cell lining of the vasculature or by foreign or other negatively charged particles, resulting in a cascade of proteolytic activation events. This coagulation cascade involves several reactions in which zymogens of serine proteases are sequentially activated, ultimately resulting in the formation of thrombin, which deposits fibrin and platelets to form a blood clot. All proteases involved in this intricate cascade of events play a part in maintaining the balance between coagulation and blood circulation. However, the balance seems to be mostly dependent on the activity of activated protein C (APC), thrombin, factor Xa (fXa), and factor XIa (fXIa). Disruption of the naturally occurring levels of these proteases in plasma may lead to severe disorders. Abnormal activities of these coagulation factors have been associated with conditions such as hemophilia, thrombosis, disseminated intravascular coagulation, Alzheimer's disease, sepsis, stroke, cancer, multiple sclerosis, and COVID-19. Despite major progress in understanding the coagulation system, the individual roles of APC, thrombin, fXa, and fXIa in various conditions have not yet been fully established, nor are there any sufficiently sensitive and selective methods to detect their individual levels in biological samples, such as blood plasma. Therefore, the development of selective chemical tools to enable coagulation factors in human plasma to be detected and discriminated would aid in unveiling their individual roles in various states of diseases.

The many roles of APC, thrombin, fXa, and fXIa in both physiological and pathophysiological processes reinforce the importance of monitoring their activity in biological samples as pharmacodynamic and diagnostic/prognostic markers. Therefore, the goal of this study was to develop potent and selective chemical tools for the simple and straightforward labeling of APC, thrombin, fXa, and fXIa in biological samples. To determine the substrate specificity of these coagulation factors, a defined P1 library and the Hybrid Combinatorial Substrate Library (HyCoSuL) were utilized. Both libraries contained a large pool of unnatural amino acids, which allowed for the chemical space in the P4-P1 positions to be more extensively explored. The library screening results enabled to design and synthesize active and selective APC, thrombin, fXa, and fXIa substrates with the ability to distinguish these proteases from other coagulation factors. The most selective substrates were then converted into

inhibitors and activity-based probes (ABPs) with Cy3/Cy5/Cy7/BODIPY fluorophores for fast, direct, and simultaneous detection of APC, thrombin, fXa, and fXIa. Finally, it was demonstrated that fluorescent ABPs could selectively label coagulation factors using mixtures of purified enzymes and human plasma. These probes can be used in the future to visualize and compare the levels of APC, thrombin, fXa, and fXIa in physiological and pathophysiological samples. The ability of these fluorescent ABPs to selectively detect these proteases in biological samples is of great importance since they serve as diagnostic and prognostic markers for multiple disorders. In the future, the set of fluorescent ABPs may prove useful in helping to unveil coagulation factors functions in health and disease. These ABPs may also be used as diagnostic tools and facilitate the choice of appropriate therapy for several disorders, such as bleeding, thrombosis, stoke, sepsis, and many others.