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ABSTRACT

The regulation of coagulation cascade plays an important role in maintaining hemostasis and any aberration in the hemostasis may lead to a hemorrhagic or prothrombotic state. An elevated prothrombotic state is a major risk factor for venous thromboembolism, atrial fibrillation and cardiac stroke. Anticoagulant therapy involving drugs that primarily target either the procoagulant proteases or regulate the endogenous anticlotting factors are used as the first line of therapy in treating hypercoagulable conditions. Antithrombin (AT) is one of the most critical endogenous regulators of hemostasis that exerts its anticoagulant activity by regulating two main procoagulant factors: factor Xa and thrombin. However, AT alone is a poor inhibitor of these proteases and requires activation by its cofactor, heparin or heparan sulfate. Further, it has been documented that, heparin binding causes exposure of certain exosite residues, which are otherwise present in an unfavorable position. Heparin binding and the concomitant exposure of the exosite residues together let AT to achieve physiologically required rates of protease inhibition. Thus, overall heparin dramatically increases the inhibitory rates of AT-protease reactions by several thousand folds. This underlies the basis of the widespread use of heparin as an anticoagulant drug in the management of thrombotic diseases like DVT & PE and also during surgical procedures. However, despite the efficiency of heparin, its continuous use is weighed down by various limitations that arise on account of serious side effects caused by heparin therapy. This necessitates the development of alternative antithrombin based anticoagulant agents with a better pharmacologic profile.

We examined the interfaces of antithrombin-protease complexes for identifying the key residues involved in each complex formation that may be utilized as putative targets to selectively modulate AT's activity against its proteases. Additionally, the theoretical analysis of antithrombin-protease complexes would give us an insight about AT's selectivity and specificity in binding to its multiple targets. On this note we directed to design non-heparin based activators of antithrombin with potent anticoagulant activity. This was initially achieved by *in silico* screening of putative molecules that can bind to AT using molecular docking approach followed by the synthesis of hit compounds and determining their AT activation and anticoagulant efficacy. We chose compounds from different scaffolds for screening and also designed the corresponding per-sulfated derivatives of various polyolic compounds. The rationale of designing sulfated compounds was to mimmic the primary structural unit of heparin, which is a highly anionic molecule with numerous sulfate groups that interacts with AT. Molecular docking identified various scaffolds with plausible binding affinity with AT. Autodock 4 based docking indicated a

sulfation induced switch in the specificity of ligands, where most of the sulfated ligands showed appreciable affinity with the HBD of AT, while the non-sulfated parent compounds docked at different surface cavities. Based on these results we selected eight compounds (from two different backbones-flavonoids and sugars) for synthesizing their polysulfated derivatives. Here we report the synthesis of three novel polysulfated compounds viz. Diosmin 2",2"',3',3",3"',4",4"',5-*O*-octasulfate, Trehalose 3,3',4,4',5,5',6,6'-*O*-octasulfate & Mannose 2,3,4,5,6-*O*-pentasulfate and one previously reported Quercetin 3,3',4',5,7-*O*-pentasulfate. Structural characterization of these compounds was carried out by various spectral studies including: FTIR, ^{1}H-NMR, ^{13}C-NMR and ESI-MS analysis. Next we examined these synthesized sulfated compounds for their ability to modulate AT's inhibitory activity against factor Xa. We observed that at physiologically permissible concentrations (5 mM each), these sulfated compounds moderately activated the AT's reactivity with fXa, that was evident from the increase in second order rate constant (k_{app}) of the AT-fXa reactions in the presence of these compounds. Trehalose sulfate and mannose sulfate were also observed to decrease the SI of antithrombin from 1 to 0.6 & 0.8 respectively, indicating an increase in rate of loop insertion due to these compounds. Next, we determined the anticoagulant potency of the sulfated compounds identified as leads from their ability to modulate AT's inhibitory activity against fXa. The effect of both sulfated leads and non-sulfated parent compounds on coagulation pathways was primarily checked by evaluating the three classical clotting assays that assess the individual coagulation pathways viz. activated partial thromboplastin time (for intrinsic pathway) and prothrombin time (for extrinsic pathway) and thrombin time (final step of coagulation). The results showed that non-sulfated compounds were ineffective in modulating coagulation both at physiologically feasible concentration and at higher concentrations. However, the effect of polysulfated compounds on these clotting tests identified our synthesized compounds; diosmin 2",2"',3',3",3"',4",4"',5-*O*-octasulfate, trehalose 3,3',4,4',5,5',6,6'-*O*-octasulfate & mannose 2,3,4,5,6-*O*-pentasulfate as having potent dose-dependent anticlotting property. The effect of the lead compounds was checked in a thrombosis model to find out the correlation between their *in vitro* and *in vivo* activity. Trehalose 3,3',4,4',5,5',6,6'-*O*-octasulfate and quercetin 3,3',4',5,7-*O*-pentasulfate were found to have a pronounced antithrombotic effect. On comparing the thrombus isolated from the thrombus induced rats treated by trehalose per-sulfate & quercetin per-sulfate with that of the untreated rat, a considerable effect on thrombus formation as well as blood clotting was observed. Further, similar to the effect as *in vitro* clotting tests the *in vivo* effect was also found to be dose-dependent. Additionally, we were interested in studying the effect of the lead compounds on platelet aggregation. Since, an anticoagulant agent with a dual mechanism of action is always more prudent as it is expected to regulate a hypercoagulable state by employing a multi-target strategy. In this venture, we analyzed the antiplatelet effect of lead compounds: Trehalose 3,3',4,4',5,5',6,6'-*O*-octasulfate and quercetin 3,3',4',5,7-*O*-pentasulfate on the platelet aggregation of whole blood isolated from animal models treated with these compounds. These polysulfated lead anticoagulant compounds were found to decrease impedance of platelet aggregation as well, suggesting a dual role of these compounds in retarding coagulation and thrombus formation.