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DISCOVERY AND BIOPHYSICAL CHARACTERIZATION OF ALLOSTERIC INHIBITORS OF FACTOR XIa (FXIa)

Malaika Argade
Virginia Commonwealth University

DISCOVERY AND BIOPHYSICAL CHARACTERIZATION OF ALLOSTERIC
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A th4sis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

by

MALAIKA D. ARGADE
Bachelor of Pharmacy, University of Mumbai, India, May 2010

Director: Dr. UMESH R. DESAI
Professor, Department of Medicinal Chemistry

Virginia Commonwealth University
Richmond, Virginia
August, 2012

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good wishes. Lastly, I cannot thank my parents and my brother, Akshay enough for being a

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Table of Contents

List of Tables

List of Figures

Abstract

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Thrombosis is one of the leading causes of mortality and morbidity that is associated with myocardial infarction, stroke and pulmonary embolism. Anti-thrombotic agents which intend to reduce the occurrence and severity of thrombosis usually target the enzymes of the coagulation cascade. FXIa, a 160 kDa homodimer is gaining popularity of late as a potential target for anti-thrombotic agents due to its relative safety.

A number of inhibitors which target the active site of FXIa have been reported but to our knowledge there have been no inhibitors which act via an allosteric mechanism. The aim of this project was to screen for allosteric inhibitors of FXIa from of pool of sulfated small-molecules.

These molecules were primarily designed to act as heparin mimetics; heparin being a natural

anti-coagulant. These compounds were then analyzed to determine whether inhibition was via an

allosteric mechanism.

Chapter 1: Introduction

1.1 The Coagulation Cascade – Intrinsic and extrinsic pathways

Under normal physiological conditions, it is essential that blood circulate throughout the system in its liquid state. However, there are certain situations, such as in the case of a vascular injury wherein a stable clot or plug is formed in a mechanism involving platelets and enzymes termed coagulation enzymes. Such an injury could be mechanical, chemical or electrical and is considered as a major stimulus for initiating coagulation.[1] These enzymes are activated from their zymogen form by proteolysis at one or more peptide bonds. Most of these enzymes also require membrane surfaces and cofactors in order to bring about the activation and these reactions may be Ca^{2+} dependent or Ca^{2+} independent. The sequence of zymogen activation and its subsequent actions, which ultimately result in the formation of a seal or clot at the site of injury, was referred to as the cascade of enzyme-proenzyme transformations by Macfarlane in 1964.[2] This is now more commonly referred to as the coagulation cascade or waterfall model.[2, 3] In addition to these coagulation enzymes or pro-coagulant agents, there also exist other entities, which are anti-coagulant in nature, such as antithrombin (AT) amongst others, which help maintain and regulate a fine balance between pro and anti-coagulation processes.[3-6]

Despite this fine balance, many situations arise wherein this balance is disturbed, which may lead to excess coagulation or bleeding (hemorrhage). The former, termed thrombosis occurs when excessive clot formation prevents the proper flow of blood through a vessel. A more severe condition, thromboembolism occurs when a part of the clot is dislodged from the site of injury and causes obstruction to blood flow through the vessels at other sites. Thrombotic disorders are

the major cause of mortality and morbidity associated with myocardial infarction, stroke, deep vein thrombosis and pulmonary embolism among others. Around 576,000 new cases of the most common thrombotic conditions, pulmonary embolism and deep vein thrombosis are detected each year in the USA.[7,8] Also, patients suffering from cancer are 3 times more likely to develop thrombotic disorders.[9] Therefore, the choice of therapy for prevention of thrombotic disorders involves anti-coagulant agents.

The initiation of a procoagulant state is triggered when tissue factor (TF), an integral membrane protein that is present on the extravascular cell surfaces is exposed to peripheral blood cells and blood flow upon vascular damage. TF is expressed at high levels in cells surrounding the blood vessels such as vascular smooth muscle cells and also in brain, kidney, lung, placenta and testis. Individuals deficient in TF have not been identified and TF-deficient mice die during embryonic development.[10] Apart from injury, TF can also be in contact with blood in cases where the endothelium is activated by chemicals, inflammatory processes and cytokines.[11] TF then forms a complex with circulating factor VIIa (FVIIa) to form the extrinsic factor Xase complex in the presence of calcium ions. In the absence of TF, FVIIa has negligible enzymatic activity, hence, the formation of this complex is essential to bring about the conversion of factors IX and X to their active forms, factor IXa (FIXa) and Xa (FXa), respectively. FXa thus generated is responsible for producing picomolar concentrations of thrombin from prothrombin. Thrombin in turn brings about activation of platelets and also converts FV and FVIII to their active forms, FVa and FVIIIa, respectively.

FVIIIa then forms a complex with FIXa to give the intrinsic Xase complex on membrane surfaces that are provided by the platelets and endothelial cells. This complex activates FX at 50-

100-fold higher rate than the extrinsic Xase complex (TF-FVIIa). Meanwhile, the FVa generated forms the prothrombinase complex with FXa, which is considered to be a major activator of prothrombin. The product of this activation, thrombin activates FXIIIa, and together these catalyze the cleavage of fibrinogen to give stable polymeric fibrin. The ultimate result of the extrinsic pathway is the formation of an insoluble fibrin clot from soluble fibrinogen.[12]
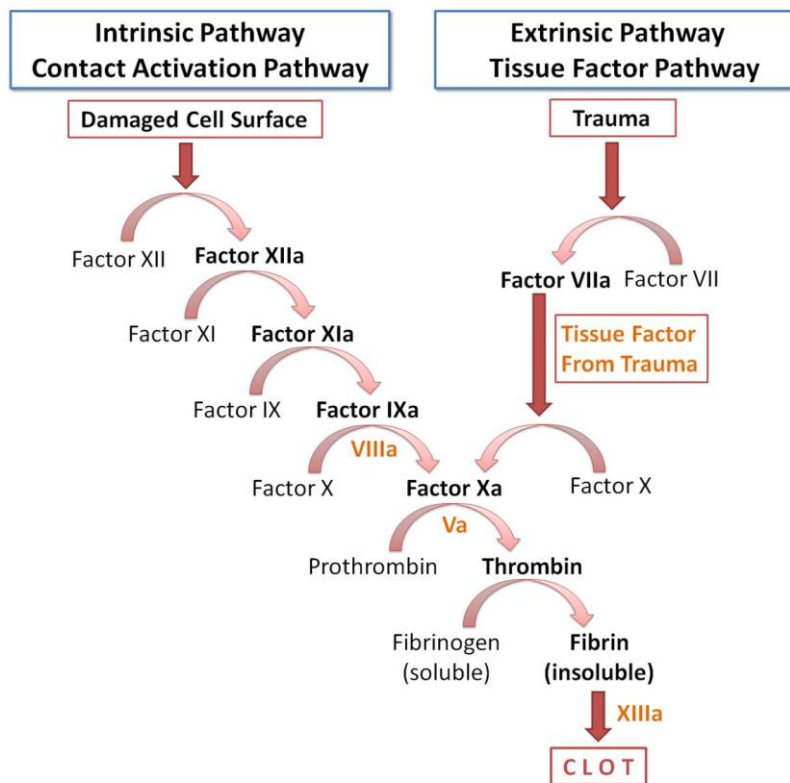
Figure 1: The coagulation cascade - Intrinsic and Extrinsic pathways

Another route for initiating coagulation is through the "contact activation" or intrinsic pathway **(Figure 1)**. This occurs when FXII or Hageman factor is activated by negatively charged surfaces and in the presence of high molecular weight kininogen and plasma kallikrein. FXIIa is also said to be activated by constituents of subendothelial matrix such as

glycosaminoglycans, sulfatides, soluble polyanions and even glass and kaolin.[13] Although activated by a number of substances, the exact mechanism of activation of FXII is uncertain.[14] Apart from the coagulation cascade, FXII is also involved in the complement system. Even though FXII was discovered in 1950s its role in these two systems remains elusive.[15]. The next step in the intrinsic pathway after activation of FXII involves formation of FXIa from FXI, which in turn activates FIX to give FIXa.

There are several entities that contribute to regulating the entire process of coagulation; one of them being tissue factor pathway inibitor (TFPI). The extrinsic Xase complex after generating a small amount of FXa is known to be inhibited by TFPI. Further generation of FXa is brought about by FIX along with its cofactor FVIII. FXa is crucial for maintaining hemostasis and it is seen that patients deficient in FIX and FVIII suffer from severe bleeding disorders showing that the presence of this complex is required for sustaining the clot through FXa formation.

A glance at the intrinsic pathway shows a linear sequence of zymogen activations, which ultimately results in the formation of activated FIX. Initially, FXIIa was thought to be the sole activator of FXI. However, emergence of studies showing that deficiency of FXII, HK and HMWK resulted in no bleeding abnormalities suggested that these initial factors were perhaps not of utmost importance in hemostasis. In contrast, deficiencies of FVIII, FIX and FXI result in bleeding disorders highlighting their importance in the formation of a blood clot. The fact that deficiency of FXI, and not FXII, PK or HMWK, caused bleeding (injury-related) led to the belief that perhaps FXI was activated by other means. It has also been shown that thrombin generated in the early stages of coagulation by the extrinsic Xase complex activates FXI, thereby ensuring

a constant supply of thrombin in a positive feedback loop. This activation is could take place in the presence of highly negatively charged surfaces such as dextran sulfate.[13, 16, 17] Also, the different bleeding phenotypes caused by deficiency of FIX and FXI shows that these two enzymes are also activated by others. Interestingly, the plasma of mammals such as whales and porpoises does not contain FXII, indicating that FXI activation by FXII is not essential for fibrin formation. These observations show that the intrinsic pathway is not linear but instead quite complicated.[18]

1.2 Traditional anti-coagulant agents

The drugs that have gained tremendous popularity as traditional anti-coagulant therapy are unfractionated heparin (UFH), low molecular weight heparin (LMWH) and warfarin.

Heparin

The word heparin is coined from the Greek word 'hepar' meaning liver, which was the tissue from which heparin was first isolated. Previously known as cephalin, this drug was first described in 1916 but became commercially available in 1940s.[18] Heparin **(Figure 2)**[8] is a highly sulfated heterogeneous and naturally occurring glycosaminoglycan (GAG) with molecular weight between 3000 -30,000 and an average molecular weight of ~15,000.
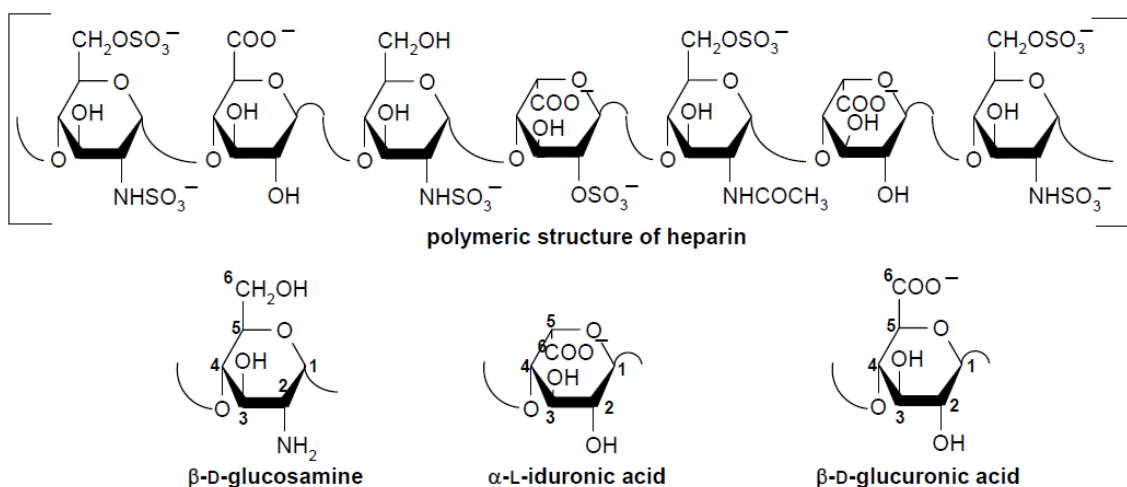
Figure 2: Heparin and its individual monosaccharide units

Antithrombin (AT) acts as an anti-coagulant by inhibiting several coagulation factors, primarily the coagulation factors Xa and thrombin (FIIa) thereby reducing clot formation. However, the rate of inhibition of these enzymes by AT is relatively slow. Heparin acts indirectly by binding to AT to give a heparin-AT complex, which shows a high affinity for thrombin and FXa resulting in a 500-4,000 fold increase in rate of inhibition. Hence, heparin can be termed an indirect inhibitor of coagulation. Although anti-coagulant therapy with heparin is tremendously popular, it has several disadvantages. Binding to AT is dependent upon a specific pentasaccharide sequence in heparin, which is seen in only one-third of heparin molecules. Heparin is very difficult to dose and monitor due to its unpredictable and variable pharmacokinetic properties.[19,21] Activated-partial thromboplastin time (aPTT), which serves as a test to measure dose effect is also found to be unreliable in this case. Apart from its anti-coagulant effect, heparin tends to interact with a number of proteins and surfaces probably due to

6

the high negative charge it possesses. This could be a major reason for a reduced bioavailability (less than 30%) when administered subcutaneously. It also binds to platelet factor 4, which gives rise to heparin-induced thrombocytopenia (HIT) which is one of the most severe complications associated with heparin therapy.[21,22]

Low-Molecular Weight Heparin (LMWH)

LMWHs are highly sulfated GAGs with a molecular weight in the range of 3,000 – 5,000. LMWHs (**Figure 3**)[8] are approximately one-third the length of UFH and contain an average of 15 monosaccharides per chain. LMWHs are prepared from UFH by chemical or enzymatic depolymerization.[22]

$X = -SO_3^-$ or $-H$; $Y = -SO_3^-$ or $-COCH_3$;
$Z = -SO_3^-$ or $-H$; $W = -SO_3^-$ or $-H$
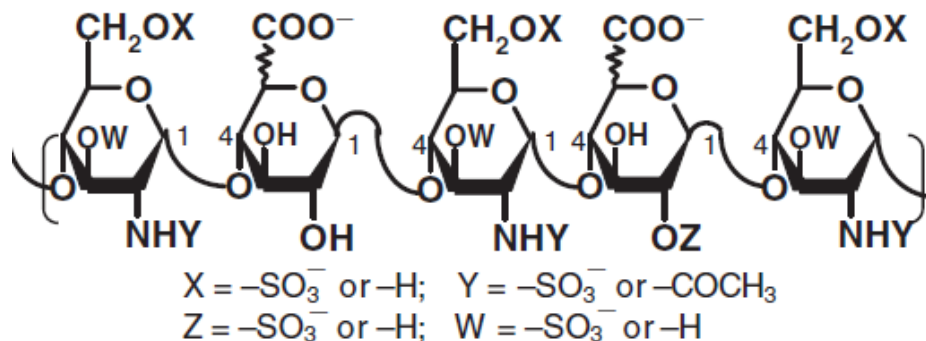
Figure 3: Structure of LMWHs M$_r$ = 5000 Da
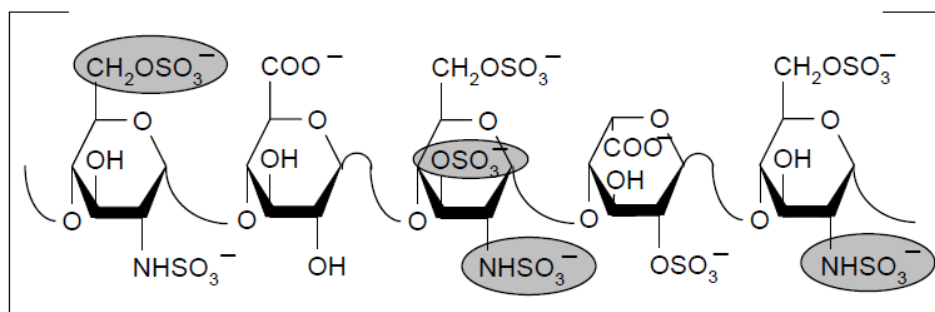
Figure 4: Pentasaccharide sequence and the negatively-charged groups (encircled) which are essential for activation and binding to antithrombin with high-affinity

Like UFH, LMWHs also bind to AT and thrombin because of the pentasaccharide sequence **(Figure 4)**.[8] However, inhibition of thrombin requires at least 13 saccharide units in addition to the pentasaccharide sequence for the formation of a ternary complex of AT, thrombin and UFH/LMWH. As a result, in comparison to UFH, LMWH has fewer chains of sufficient length, which can aid in inhibiting thrombin. LMWHs comparatively have predictable anti-coagulant response because it has lesser propensity to bind to other proteins and surfaces. Due to different molecular weights and anionic character the effect of LMWHs and UFH is different, with the former having a bioavailability of more than 90% when administered subcutaneously. However, LMWH effect cannot be reversed by administration of protamine. Also the elimination of drug is by renal clearance and hence is not safe for patients suffering from renal insufficiency.[22]

Warfarin

First introduced as a rat-poison, coumarin derivative warfarin **(Figure 5)** gained popularity as an anti-coagulant drug due to its high water solubility and oral bioavailability in

contrast to UFH and LMWH which required parenteral administration.[18] It acts by reducing the synthesis of vitamin-K dependent coagulation factors II, VII, X, IX and anticoagulant protein C and S.
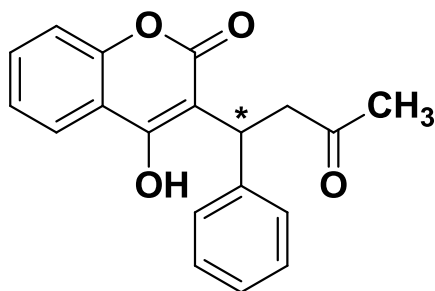
Figure 5: Structure of oral anti-coagulant, Warfarin

However, the major disadvantage of warfarin is its narrow therapeutic index and its tendency to interact with vitamin-K rich foods and other drugs. Also, warfarin is contraindicated in pregnancy as it causes bleeding and teratogenecity. Warfarin has a high tendency to cause bleeding and therefore in 2006 the FDA put a "black box" warning on the drug.[8]

Direct Thrombin Inhibitors (DTIs)

Hirudin, **(Figure 6)** which was isolated from the leech *Hirudo medicinalis* in 1884, is said to be the most potent direct inhibitor of thrombin with a K_i value of about 20 fM. However, hirudin possesses a major risk of bleeding episodes and has a very narrow therapeutic index. Apart from this, its high potency for thrombin gives rise to complexes which are very tightly bound and therefore irreversible. This poses a problem as there is no antidote available which can reverse the complexes that are formed. Hirudin also has immunogenic capabilities as it is a foreign peptide. To reduce the risks of bleeding associated with hirudin, other DTIs such as

bivalirudin **(Figure 6)** and argatroban were introduced. However, these still carried a significant risk of bleeding. Another disadvantage of DTIs is their poor oral bioavailability, which is due to the presence of cationic arginine, guanidine or amidine groups in these molecules.[8]

Residue	63	64	65
Hirudin	Tyr^{63}-OSO$_3^-$	Gln	Leu
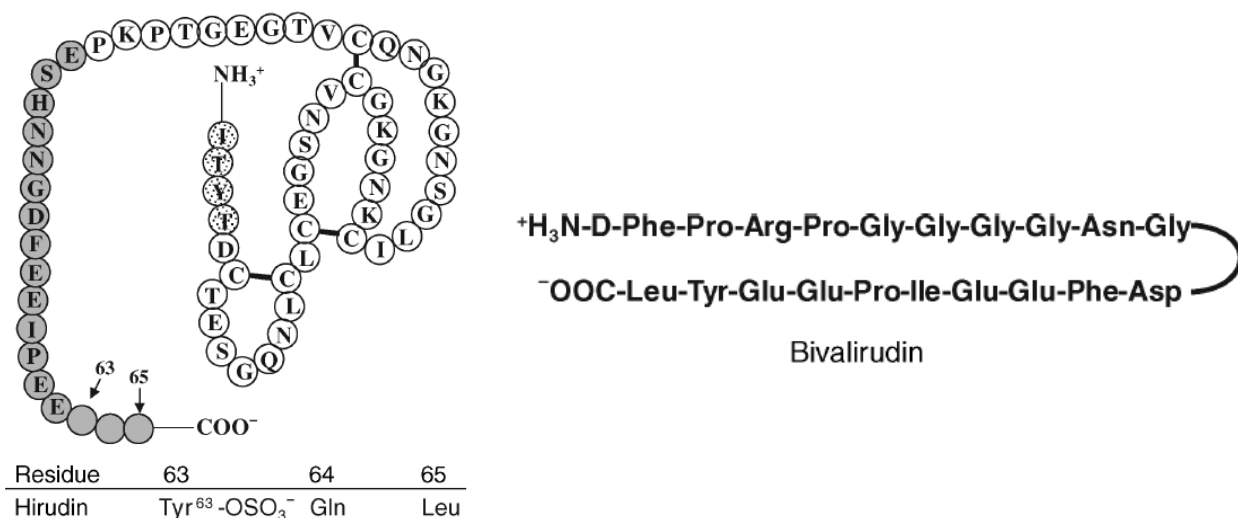
Figure 6[8]: Structures of hirudin and bivalirudin, DTIs

Factor Xa Inhibitors

These are of two kinds, direct and indirect inhibitors. The direct FXa inhibitors include rivaroxaban **(Figure 7)**, which is a selective, oral and competitive inhibitor.
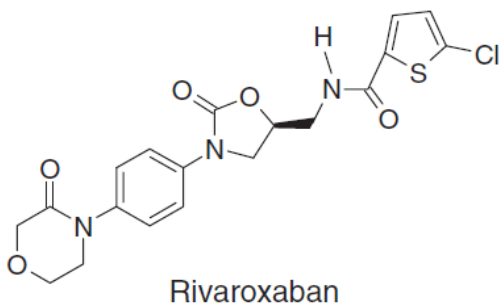
Rivaroxaban

Figure 7[8]: Structure of rivaroxaban

Fondaparinux and idraparinux (**Figure 8**) are examples of indirect (AT dependent) factor Xa inhibitors and are synthetic analogs of the pentasaccharide sequence that is essential for the interaction of heparin with AT.[8,20]

(a)

(b)
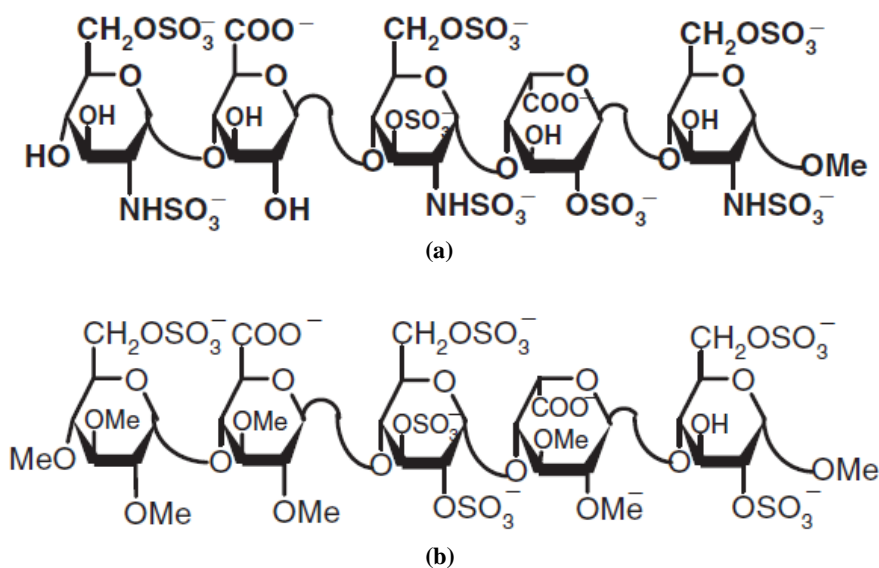
Figure 8[8]: Structure of indirect Factor Xa inhibitors (a) Fondaparinux, (b) Idraparinux

1.3 Emergence of FXIa as a target

The disadvantages associated with the traditional anti-coagulant drugs mostly stem from the fact that these drugs target enzymes that are required for hemostasis. Targeting such enzymes is prone to adverse consequences. This has led to a growing need for newer drugs that work through safer targets. Such a target should have a distinguishing role in the processes of hemostasis as well as thrombosis.

From the small molecule perspective, an ideal anti-coagulant would be the that reduces thrombosis without disturbing normal hemostasis of the system. Hence, targeting the enzymes of

the intrinsic pathway is gaining interest as these are said to play a major role in thrombus formation and a minor insignificant role in hemostasis. Of the intrinsic pathway enzymes, FXI and FXII emerged as attractive targets in anti-thrombotic therapy.[23-26] However, as previously mentioned, the role of FXII remains elusive and its involvement in not only the coagulation system but also prekallikrein-kinin, fibrinolysis and complement system makes it an unsafe target due to the increased risks of adverse effects.[27]

In contrast to the other well-known haemophilias, deficiency of FXI (Haemophilia C) is found to be relatively mild and is found to be most prevalent in Ashkenazi Jews. Spontaneous bleeding or hemorrhaging is rare in patients with FXI deficiency, with bleeding occurring only after a surgery and in locations of high fibrinolytic activity such as oral cavity, nose, tonsils and urinary tract.[28,29] Since the deficiency of FXI does not cause very severe bleeding disorders as are seen with deficiencies of other clotting factors, its appears relative safety as a target.

In the revised model of blood coagulation, thrombin generated in small amounts in the early stages of coagulation ensures its generation by activating FXI. Hence, FXIa is responsible for rapidly generating thrombin required for maintaining the fibrin clot integrity.[13] In order to study the effect of FXI deficiency on thrombin generation in vivo, several mice studies were conducted. In a particular study, an injury on the carotid artery was brought about by using $FeCl_3$ in both, wild-type and FXI deficient mice. Ferric chloride tends to injure the vascular endothelium, thereby exposing collagen to circulating blood. It was seen that the injury caused the formation of an occlusive thrombus within 5-14 mins in contrast to the FXI-deficient mice in which the blood flow was minimally restricted, the flow decreasing gradually over 60 mins to 50-70% of the rate before injury. The effect of FXI deficiency on clot formation in mice was

found to be more severe than seen in mice deficient in FVII or FIX. Interestingly, the ability to form clots was restored in the FXI-deficient mice following injection of human FXI. It is also seen that FXI deficiency provides some level of protection against thrombus formation in the carotid artery of mice. In a similar $FeCl_3$-induced injury study, the protective effect of FXI-deficiency in mice against arterial thrombosis was compared with those achieved by administering heparin and aspirin. It was seen that FXI-deficiency achieved protection equivalent to those produced by a very high heparin dose (1000 units/kg) and aspirin (30 mg/kg).[30,31] Similar results were obtained in studies conducted in mice to determine effect of FXI-deficiency on $FeCl_3$-induced vena cava thrombosis.[32] In the baboon model, it was seen that FXI inhibition reduced thrombus growth rate, thrombo-occlusion and overall mass of the thrombus. Hence, this shows that FXI is a good target for antithrombotic therapy.[33] Another study on the propagation of thrombus formation in atherosclerotic plaque concluded that FXI is not only involved in the thrombi but also contributes to its formation. Therefore, when FXI was inhibited with a monoclonal antibody, the thrombus growth was reduced but there was no significant change in the bleeding time.[34] All these results show that inhibition of FXI could help in preventing thrombosis without the side effect of bleeding.

FXI is said to play an essential role in thrombin generation and there is a large body of work which proves that this contribution is due to a feedback activation mechanism by thrombin and is independent of FXIIa.[35-37] The thrombin generated by FXIa is said to be responsible for activating TAFI (thrombin-activatable fibrinolysis inhibitor) in a reaction that requires high concentrations of thrombin. TAFI is responsible for the down regulation of fibrinolysis by factor

XI.[38] Hence, inhibiting FXIa might make the clot more prone to dissolution since fibrinolysis will not be hampered by TAFI.

1.4 Structure and function of FXI

FXI is a unique serine protease zymogen and differs from vitamin-K dependent proteases in being a dimer of identical subunits **(Figure 9)**. It lacks a calcium binding Gla domain at the *N*-terminus, which is a characteristic of the vitamin-K dependent proteases. Each FXI subunit consists of four Apple domains (A1, A2, A3 and A4) named from the heavy chain *N*-terminal and a trypsin-like catalytic domain (CD) at the *C*-terminus. Each apple domain consists of 90-91 amino acids and are 23-34% identical among the domains. The two subunits of 80 kDa each are held together by an interchain disulfide bond at Cys^{321}-Cys^{321} and the residues Leu^{284}, Ile^{290} and Tyr^{329} constitutes the hydrophobic core in the A4 domain and are said to be essential for dimer formation. Each apple domain is made up of 7 anti-parallel β strands cradle a single α-helix. The entire arrangement of the apple domains upon the catalytic domain is sometimes referred to as the "cup and saucer" arrangement.[39-41] Like other serine proteases, FXIa has a catalytic triad which consists of His^{413}, Asp^{462} and Ser^{557}.[42] Activation of FXI is brought about by cleavage of the peptide bonds, Arg^{369}-Ile^{370} by FXIIa and other studies have also shown that activation can be brought about by thrombin and by FXIa (autoactivation).[39] FXI is also reported to interact with surfaces of activated platelets and this interaction is essential for activation of FXI. The residues on FXI which are said to play a role in this interaction are Arg^{250}, Lys^{255}, Phe^{260} and Gln^{263} which are found to be located on the apple-3 domain of FXI.[43]

(**Figure 9** was generated using the software The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. PDB ID: 2F83)
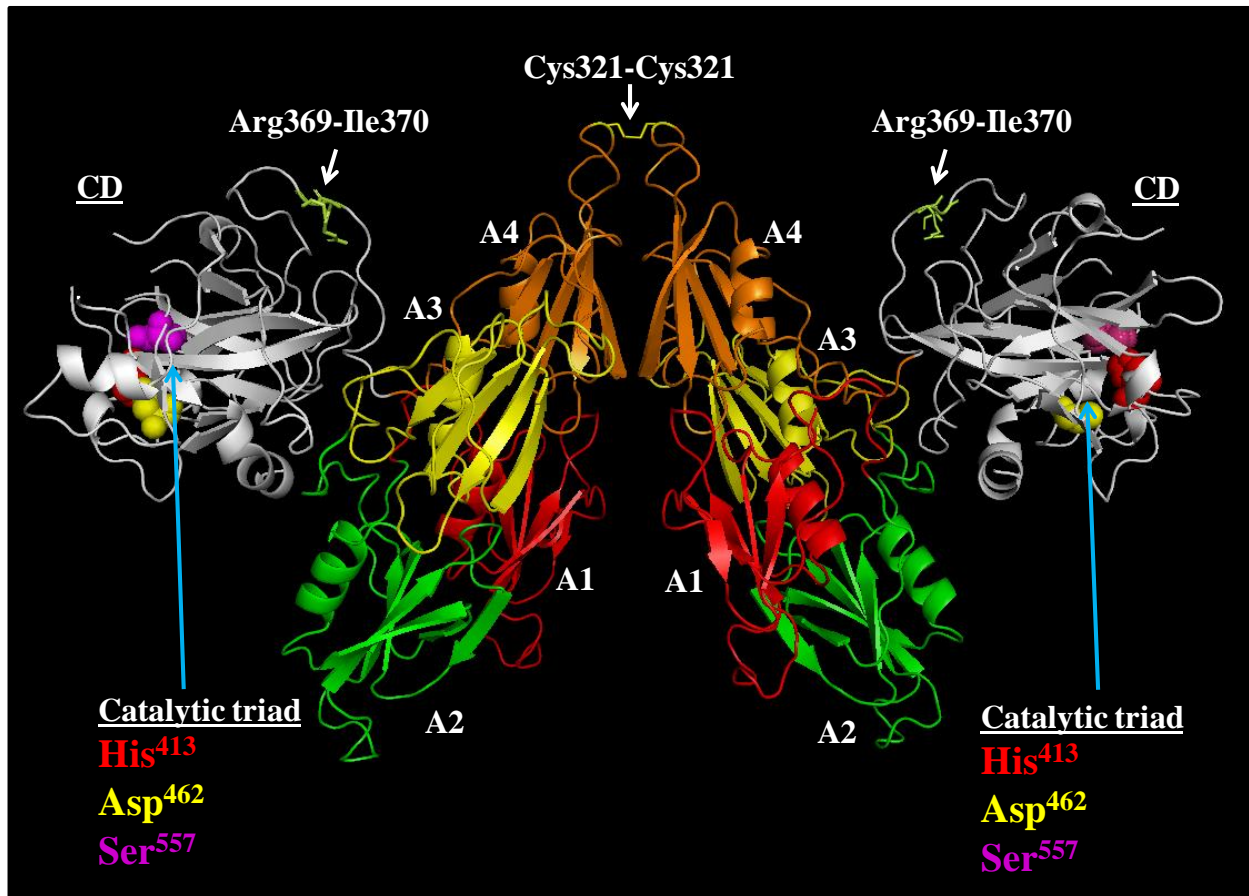
Figure 9: Factor XI showing apple domains (A1, A2, A3 and A4) and catalytic domains.

The cleavage in each subunit during the activation process gives rise to a heavy chain made up of 369 amino acids and a light chain (catalytic domain) made up of 238 amino acids. Out of the four apple domains A1 is said to be essential for binding of HMWK. The natural substrate for FXIa is FIX, which is said to bind to exosites other than the active site on FXIa, which are otherwise not exposed in the inactive form, FXI. Studies have shown a FIX-binding

site on the A3 domain and a critical residue for activation of FIX is Arg[184]. In addition the A4 domain is also said to bind FXIIa.[39]

The crystal structure of the zymogen FXI purified from human plasma shows that the two saucers (apple domains) are inclined at an angle of 70° and gives rise to an inverted 'V' shape with the active sites pointing away. This structure revealed that the A2 domains of each subunit are spread apart while the A1 and A3 of adjacent domains are in close contact.[44]

1.5 Inhibitors of Factor XIa

Even though the newer drugs targeting specific enzymes (recent ones being thrombin and FXa) are better than traditional therapy, they still fail to overcome bleeding problems and have about the same efficacy-to-safety index as heparin and warfarin. The first crystal structure that was obtained of FXIa was with certain ecotin mutants. Ecotin is secreted by *Escherichia coli* and is a pan-serine protease protein inhibitor of 142 amino acids. A group of ecotin mutants were prepared and their crystal structures with a mutant FXIa were studied in order to understand the way in which a substrate could bind to FXIa.[45] These results gave information that could prove to be important in drug design of potential FXIa inhibitors in the future. However, it was difficult to obtain a crystal structure of a small molecule active site inhibitor with wild-type FXIa. Finally, after making certain mutations on 4 residues on the surface of FXIa a crystal structure of FXIa with benzamidine was obtained. Benzamidine is a weak active site inhibitor with an IC_{50} of 100 μM. Benzamidine is reported to bind at the S1 pocket when its amidine group forms a hydrogen bond with Asp[189] and also the carbonyl oxygen of Gly[218].[46]

Another inhibitor of FXIa is clavatadine A. Out of ~39,000 biota extracts that were screened against FXIa, one showed inhibition of the enzyme with an IC_{50} of around 0.4 g/L. Fractionation of this interesting extract (obtained from marine sponge *Suberea clavata*) led to the discovery of two bromophenol alkaloids, which were named as clavatadine A (1) and (2) whose structures are shown in **figure 10**. These did not inhibit FIXa and are reported to be selective, irreversible inhibitors of FXIa. The IC_{50}s of the compounds (1) and (2) for FXIa were found to be 1.3 and 27 µM respectively.[47]
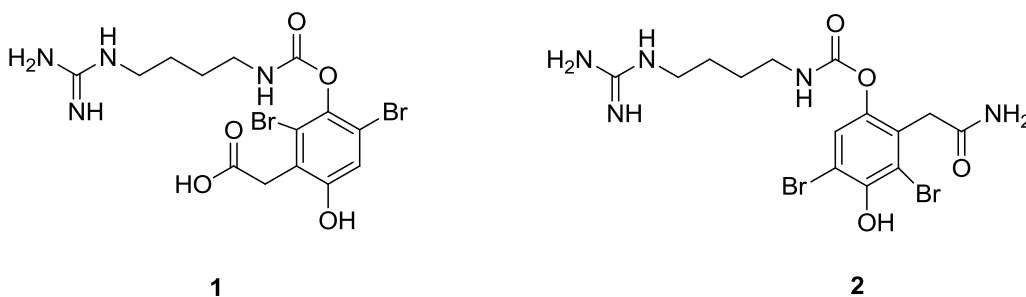
Figure 10: Clavatadine A (1) and (2)

The carbamate moiety of (1) is reported to form a covalent bond with the active site serine residue while the guanidine binds to Asp[189] and Gly[218]. A mutant of FXIa was prepared at residues S[434]A, K[437]A, T[475]A and C[482]S in order to improve crystallizability.[47] The crystal structure of clavatadine A (1) with FXIa is shown **(Figure 11)** and shows the carbamate side chain of 1 forming a covalent bond with the active site Ser[195].
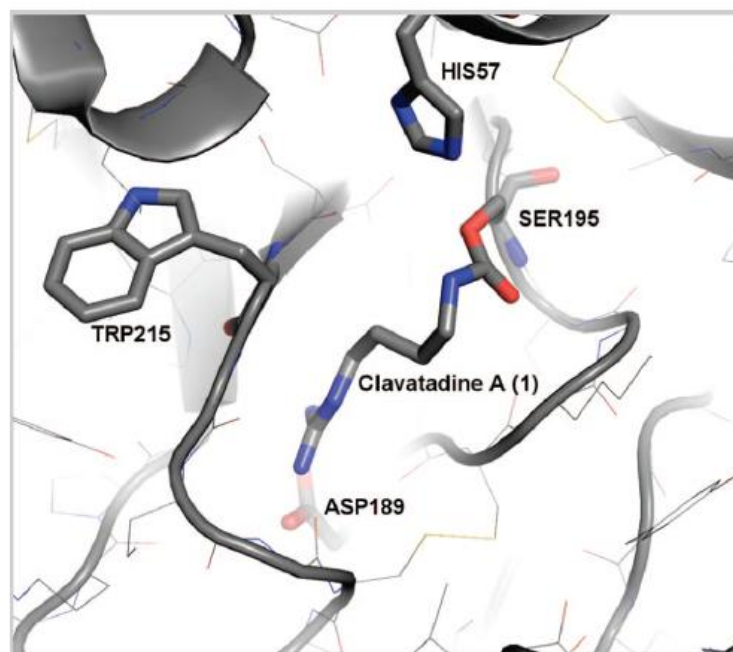
Figure 11: Crystal structure of clavatadine A (1) with FXIa.

(Figure adapted from- Buchanan, M. S.; Carroll, A. R.; Wessling, D.; Jobling, M.; Avery, V. M.; Davis, R. A.; Feng, Y.; Xue, Y.; O¨ster, L.; Fex, T.; Deinum, J.; Hooper, J. N. A.; Quinn, R. J. Clavatadine A, A Natural Product with Selective Recognition and Irreversible Inhibition of Factor XIa. *J. Med. Chem.* **2008**, *51*, 3583-3587.)

A structure activity study was carried out for FXIa by screening derivatives of aryl boronic acid inhibitors for FXIa. Simple or substituted aryl boronic acids are said to weakly inhibit certain serine proteases. For screening, commercially available aryl boronic acid derivatives were selected which had a substituent which could act as a hydrogen bond donor. This would help in forming an electrostatic interaction with Asp[189] of the FXIa active site. Clavatadine A (**1**) and (**2**) did inhibit FXIa with an IC_{50} of 77.3 and 120 μM respectively. Clavatadine A (**1**) was chosen for further structure activity relationship studies. In order to

18

improve the interaction of the molecule with the Asp[189] residue at the active site, a guanidinium group was introduced instead of the methylene amine and the linker between the guanidium and phenyl ring were varied in length in order to explore the effect on interaction with Ser[195] and also with Asp[189]. The compounds were synthesized in the form of borate pinacol esters.
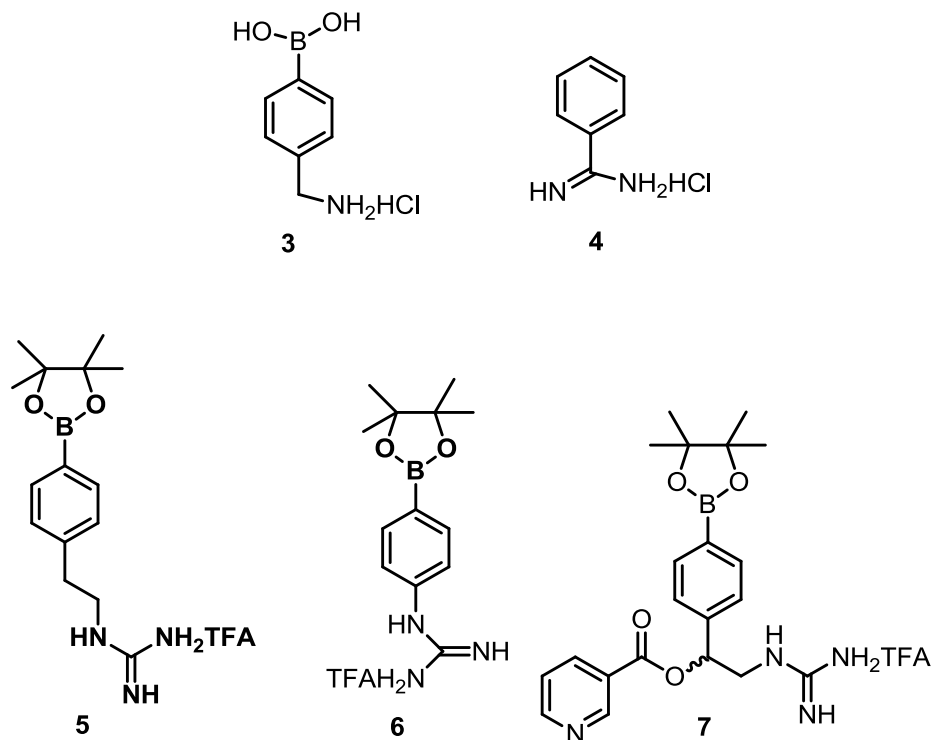
Figure 12: Aryl boronic acid derivatives

The interaction of these molecules (**Figure 12**) with the active site was studied by obtaining the crystal structures of these molecules in a mutant FXIa, rhaFXI$_{370\text{-}607}$-S434A, T475A, C482S and K437. The crystal structures of these molecules showed that the boron atom formed a covalent bond with the active site Ser[195] residue and was tetracoordinate. Also, the guanidine group in these molecules was found to interact with aspartate 189 which is present in the S1 pocket. The binding of these three molecules was quite similar except for the phenyl ring

19

of compound **12**. This led to the inference that modification of substitution at the meta position on the phenyl ring could help in reaching a small pocket on the S1 site of FXIa. This gave rise to synthesis of a molecule **7** with a chiral center which had an IC_{50} of 1.4 µM and a greater selectivity against trypsin compared to its precursor, compound **5**.
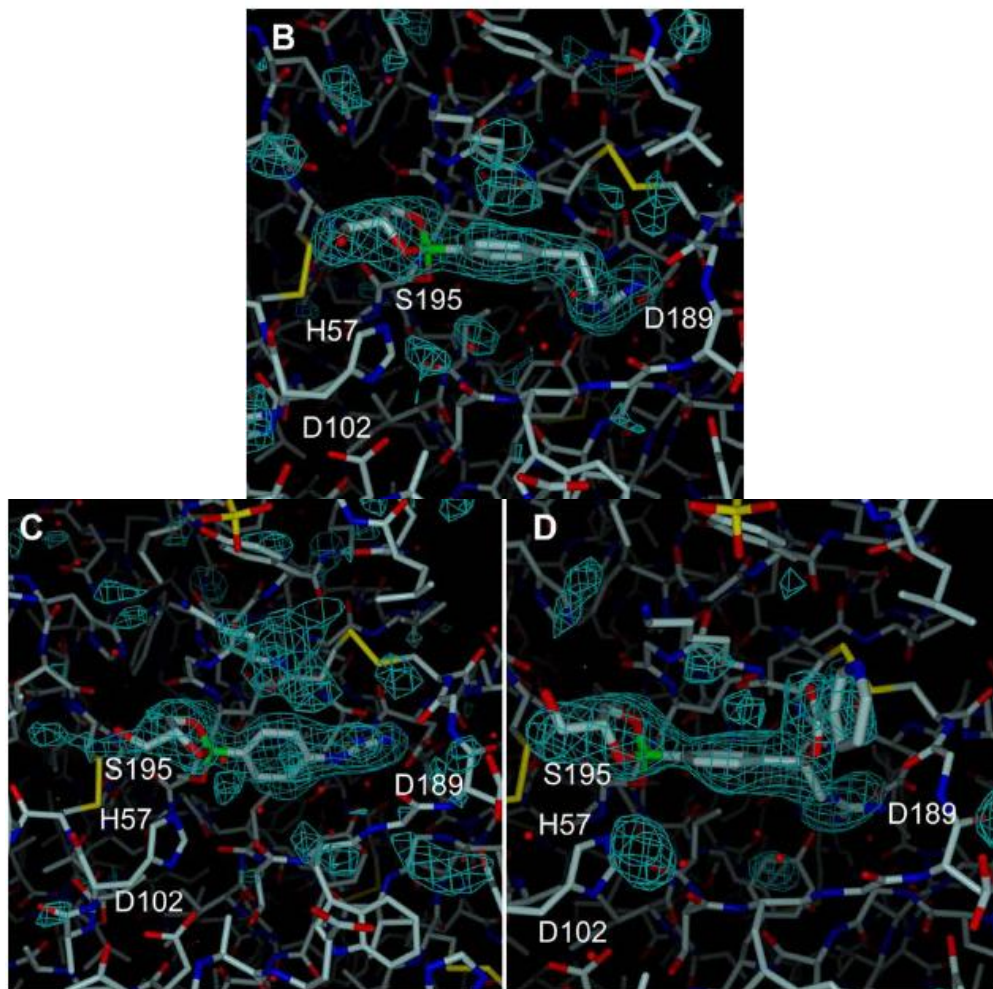
Figure 13: Electron-density maps B, C and D for FXIa and compounds 10, 12 and 13 respectively

(Figure adapted from- Lazarova, T. I.; Jin, L.; Rynkiewicz, M.; Gorga, J. C.; Bibbins, F.; Meyers, H. V.; Babine, R.; Strickler, J. Synthesis and in vitro biological evaluation of aryl boronic acids as potential inhibitors of factor XIa. *Bioorg. Med. Chem. Lett.* **2006**, *16,* 5022-5027.)

Although the crystal structure of this molecule with FXIa (**Figure 13**) showed similar interactions, both molecules **10** and **13** bound in a different manner. Compound **13** exhibited additional interactions with lysine 192 and leucine 146. These interactions especially the one between leucine 146 and the pyridyl group of **13** could be responsible for its greater selectivity as compared to compound **10**.[48] Apart from the above mentioned compounds, there have also been certain peptidomimetic and non-basic synthetic inhibitors that were tested against FXIa.[49,50]

1.6 Rationale

It has been reported that the inhibition of FXIa can be brought about by highly charged polyanions such as dextran sulfate, heparin and hypersulfated heparin. It was also shown that the inhibition was brought about not by binding to the active site, but instead at an allosteric site.[51] Drugs that usually target the enzymes of the coagulation cascade tend to rely on a saccharide scaffold with the hope that these would act as heparin mimetics. However, the problems associated with saccharide based drugs are numerous such as poor bioavailability and undesired side effects due to the interaction of these highly charged molecules with other proteins or cells. Another approach to designing new drugs is to synthesize non-saccharide based sulfated small molecules. Such molecules are reported to have more advantages such as a more hydrophobic nature which could contribute to better oral bioavailability, greater synthetic accessibility and most importantly, specificity.[52] Initially, a number of molecules were synthesized successfully in

our laboratory to function as allosteric modulators of antithrombin. Over the years, our laboratory has generated a library of small sulfated non-saccharide based molecules.[52]

Although as previously described, a number of active site inhibitors have been reported, there have been no reported allosteric inhibitors of FXIa. The major aim of this project was to screen our library of molecules against FXIa with the hope of finding potential allosteric inhibitors for this particular enzyme. One of the major advantages of targeting an allosteric site is the selectivity achieved. It is observed that the active site pockets or substrate binding regions are usually conserved in the proteases that belong to a particular family. As a result, among these proteins it is difficult to find an active site inhibitor that could exhibit selectivity for any one particular protein. Hence, targeting an allosteric site will most probably provide better selectivity as compared to that for an active site.[53-55] Another advantage is the effect produced by allosteric modulators reaches saturation and hence once the sites are occupied no more effect can be observed.[56,57]

In the case of FXIa, heparin is reported to bind to sites which are rich in positively charged amino acid residues such as arginine and lysine (discussed in detail in Chapter 3). Hence, the presence of varying numbers of the sulfate groups on different locations on a small scaffold could perhaps mimic the ionic interaction that heparin has with FXIa. After identification of the potential allosteric inhibitors, further studies would involve characterizing the regions where these interact with FXIa, possibly by comparing it with known inhibitors such as heparin.

Chapter 2: Discovery of small sulfated molecules as FXIa inhibitors

2.1 Aim

The main aim at the start of the project was to screen a mini-library of small sulfated molecules in order to find small-molecule allosteric inhibitors of FXIa which may act in a fashion similar to those of the polyanions. This involved the screening of a total of 26 small molecules and determination of the IC_{50} for those molecules which showed reasonable inhibition. Upon finding the molecules of interest, experiments were conducted to determine whether the inhibition of the enzyme was due to an allosteric mechanism or competitive binding. Prior to screening, the Km of the substrate for FXIa was determined by testing in three different buffers.

2.2 Determination of Km of substrate S-2366 for FXIa.

2.2.1 Materials:

The enzyme human factor XIa was purchased from Haematologic Technologies (Essex Junction, VT) and the substrate S-2366 (*L*-pyroglutamyl-*L*-prolyl-*L*-arginine-*p*-nitroaniline) was purchased from Diapharma (West Chester, OH). All the other chemicals were purchased either from Sigma Aldrich (St.Louis, MO) or from Fisher Scientific (Pittsburgh, PA) and were of biochemical grade.

2.2.2 Procedure and results:

The Michaelis-Menten constant Km was determined at varying concentrations of S-2366 at constant concentration of FXIa. For the experiment the aliquots of the stock enzyme were made by using a buffer containing 150 mM NaCl, 50 mM Tris, 0.02% PEG 8000 and 0.1% Tween-80 at pH 7.4. The final concentration of the enzyme was 0.765 nM and was constant throughout the experiment. The concentration of the substrate varied from 1.6 mM to 0.009 mM. The three buffers that were tested were,

Buffer A: 0.09M Tris, 0.09M NaCl, 0.1% PEG-8000, 0.02% Tween-80 at pH=8.3

Buffer B: 0.09M Tris, 0.09M NaCl, 0.1% PEG-8000, 0.02% Tween-80 at pH=7.4

Buffer C: 0.05M Tris, 0.15M NaCl, 0.1% PEG-8000, 0.02% Tween-80 at pH=7.4

Each individual well contained 90 µL of buffer (maintained at 37°C) and 5 µL of enzyme FXIa. After incubating for 10 mins at 37°C in the microplate reader (Molecular Devices-FlexStation3), the substrate was added and the absorbance at 405 nm was measured immediately to determine the initial velocity of p-nitroaniline released. The velocities of the reactions were monitored on the basis of the absorbance of p-nitroaniline generated from substrate cleavage by FXIa, in individual wells. The values were obtained by using the software, SoftMax Pro Microplate Data Acquisition and Analysis software and the results were plotted using the software SigmaPlot (Systat Software, San Jose, CA) and the best fit was obtained using the formula,

$$V = \frac{[S]Vmax}{[S] + Km}$$

For all the three buffers, the values of initial velocity were plotted against the concentration of the substrate as can be seen in **figures 14, 15 and 16**.
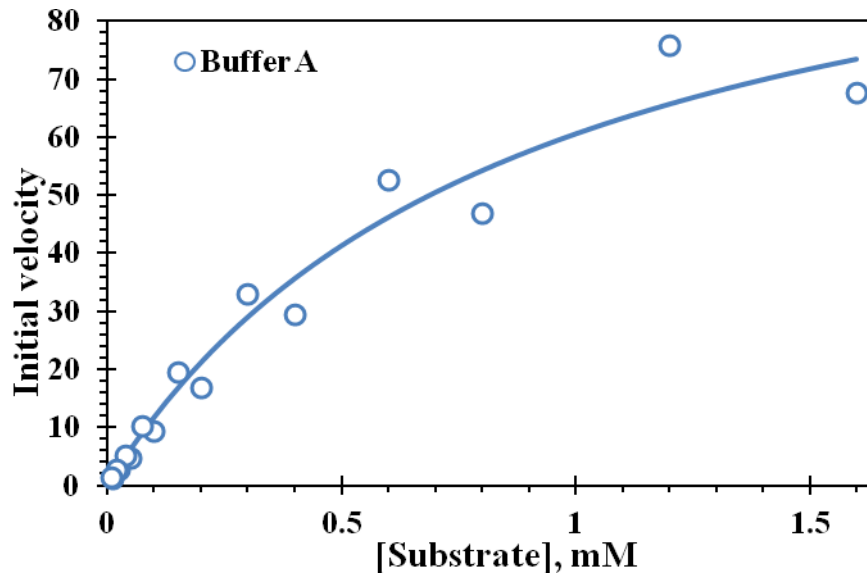
Figure 14: Determination of K_m with Buffer A
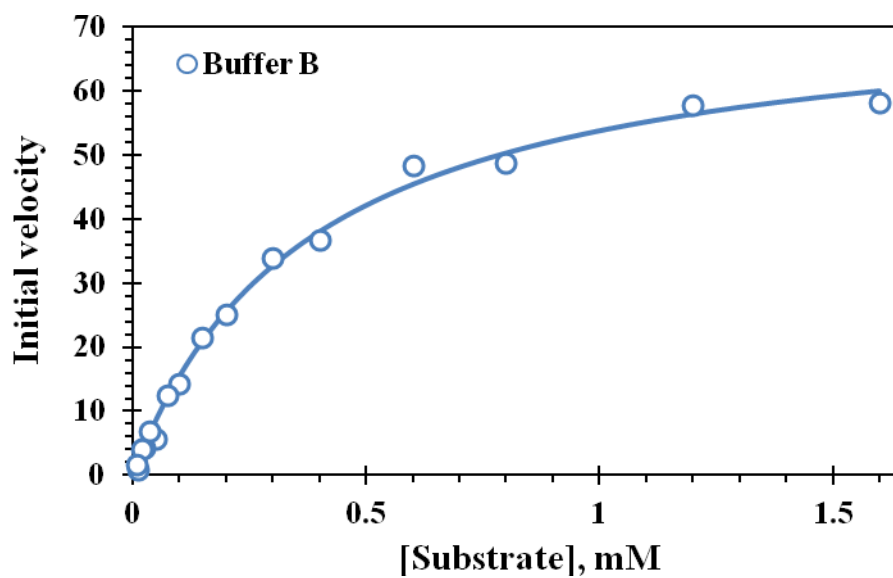
Figure 15: Determination of K_m with Buffer B
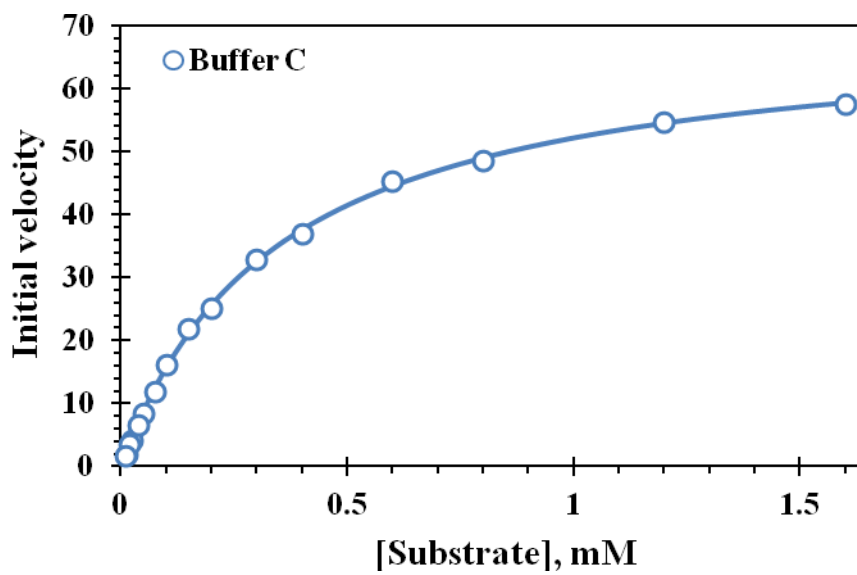
Figure 16: Determination of K_m with Buffer C

Buffer	Km (µM)	Vmax
A	880 ± 0	114 ± 14
B	382 ± 0	74 ± 2
C	348 ± 0	70.3 ± 0.6

Table 1: Results of Michaelis-Menten kinetics for compounds 13 and 24

On comparing the values of Km for the three buffers **(Table 1)**, it was seen that buffer C produced a reasonable V_{max} with a K_m that was the lowest compared to those of buffer A and B. Based on these results it was decided that buffer C would be used for screening of the library as well as other future experiments.

2.3 Discovery of FXIa inhibitors:

The mini-library consisted of small molecules of different functionalities and scaffolds and the screening was carried out by using a 96-well microplate. The compounds consisting of different scaffolds are given in **tables 2, 3, 4, 5 and 6**. The syntheses of these compounds have been reported by our group.[58-60] The compounds having a concentration of at least 300 μM (with water as solvent) were incubated with the 0.765 nM enzyme for 10 minutes at 37°C. As mentioned previously, buffer C was used for the screening and its temperature was maintained throughout at 37°C. For each column of the microplate, 2 wells were kept as blanks in which solvent (water) was added instead of an inhibitor. The remaining 6 wells of each column contained a different inhibitor. After the incubation, the substrate S-2366 was added such that the final substrate concentration in each well was 330 μM. The initial rate of the substrate cleavage by FXIa was determined by monitoring the change in absorbance of *p*-nitroaniline at 405 nm by using a microplate reader and FlexStation III (Molecular Devices, Sunnyvale, CA).

The compounds were then filtered in terms of the % residual activity of the enzyme and which is given by,

$$\% \text{ Residual Activity} = \frac{\text{Initial rate with inhibitor}}{\text{Initial rate without inhibitor (blank)}} \times 100$$

A cut-off of 50% was applied and any inhibitor which exhibited a % residual activity of less than that was further analyzed to determine their IC_{50} for FXIa.
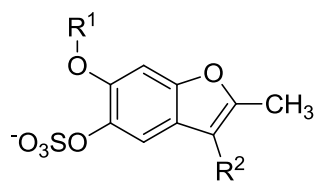
Compound Number	R^1	R^2
1	CH_3	
2	CH_3	
3	CH_3	
4	H_2C-CH_3	
5		
6		

Table 2: Sulfated benzofuran monomers

1
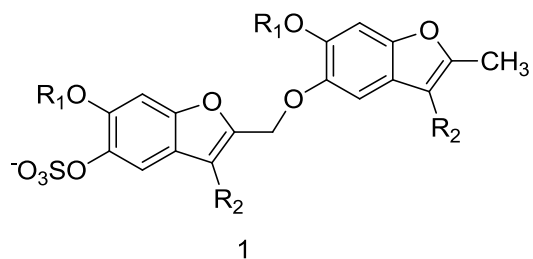
Compound Number	R^1	R^2
7	CH_3	
8	CH_3	
9	CH_3	
10	CH_3	
11	CH_3	
12	CH_3	
13	H_2C-CH_3	

Table 3: Sulfated benzofuran dimers
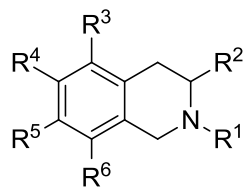
Compound Number	R^1	R^2	R^3	R^4	R^5	R^6
14	(structure with OSO$_3^-$, OSO$_3^-$, O)	COO$^-$	OSO$_3^-$	H	H	OSO$_3^-$
15	(structure with OSO$_3^-$, OSO$_3^-$, O)	COO$^-$	OSO$_3^-$	H	H	OSO$_3^-$
16	(structure with OSO$_3^-$, OSO$_3^-$, O)	COO$^-$	H	OSO$_3^-$	OSO$_3^-$	H
17	(structure with OSO$_3^-$, OSO$_3^-$, O)	COO$^-$	H	OSO$_3^-$	OSO$_3^-$	H
18	(structure with OSO$_3^-$, OSO$_3^-$, O)	COO$^-$	H	OSO$_3^-$	OSO$_3^-$	H
19	(structure with OSO$_3^-$, OSO$_3^-$, O)	H	H	OSO$_3^-$	OSO$_3^-$	H

20	OSO$_3^-$ ... OSO$_3^-$ O (structure)	H	H	OSO$_3^-$	OSO$_3^-$	H
21	OSO$_3^-$ OSO$_3^-$ O (structure)	H	H	OSO$_3^-$	OSO$_3^-$	H
22	O OSO$_3^-$ OSO$_3^-$ (structure)	H	OSO$_3^-$	OSO$_3^-$	H	H

Table 4: Sulfated tetrahydroisoquinoline scaffold

H$_3$CO, OSO$_3^-$, OC$_2$H$_5$, R$_2$O, $^-$O$_3$SO, OR$_1$ (benzofuran trimer structure with substituents)
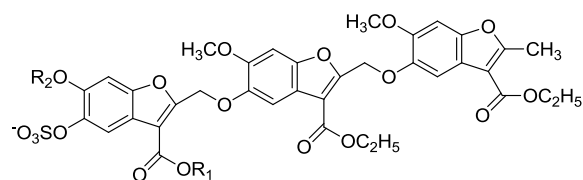
Compound Number	R^1	R^2
23	—CH$_3$	—CH$_3$
24	—C$_2$H$_5$	—C$_2$H$_5$
25	—C$_2$H$_5$	—C(CH$_3$)$_3$ (tert-butyl)
26	—HC(CH$_3$)$_2$ (isopropyl)	—CH$_3$
27	—C$_2$H$_5$	—CH$_3$

Table 5: Sulfated benzofuran trimers

32
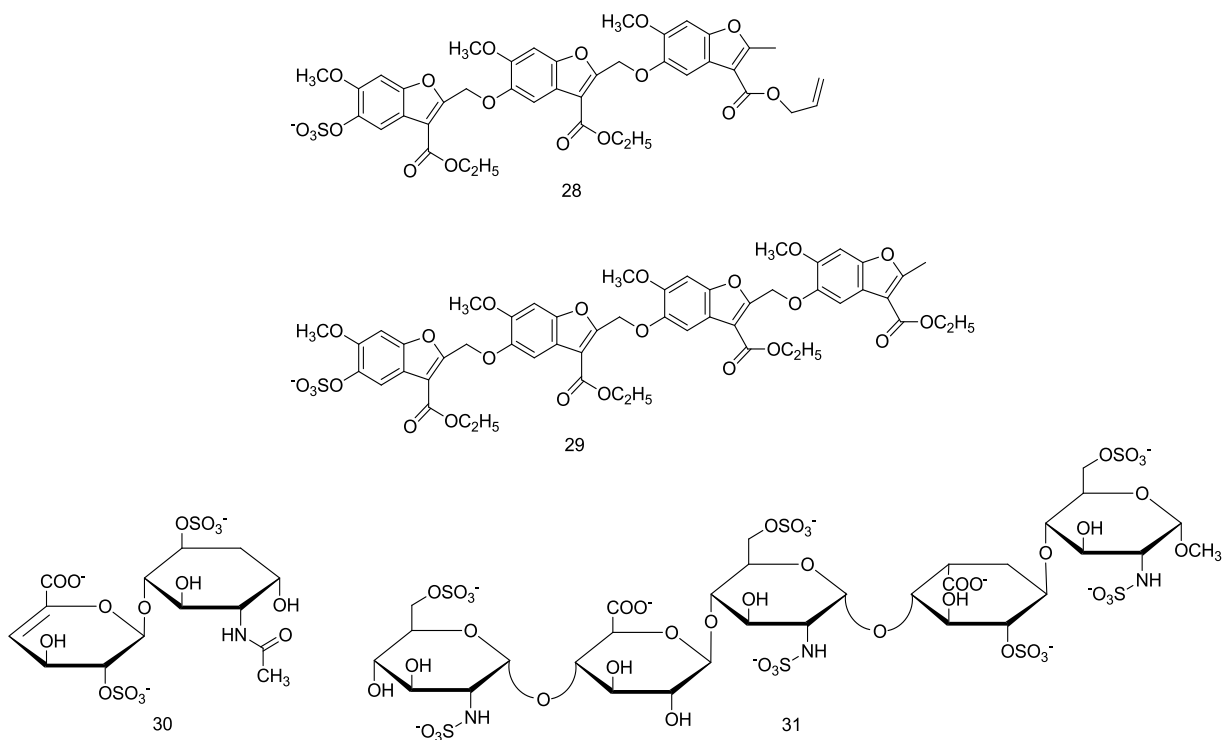
Table 6: Other sulfated small-molecules

The % residual activity for these molecules was determined and the results obtained can be seen in **figure 17**.
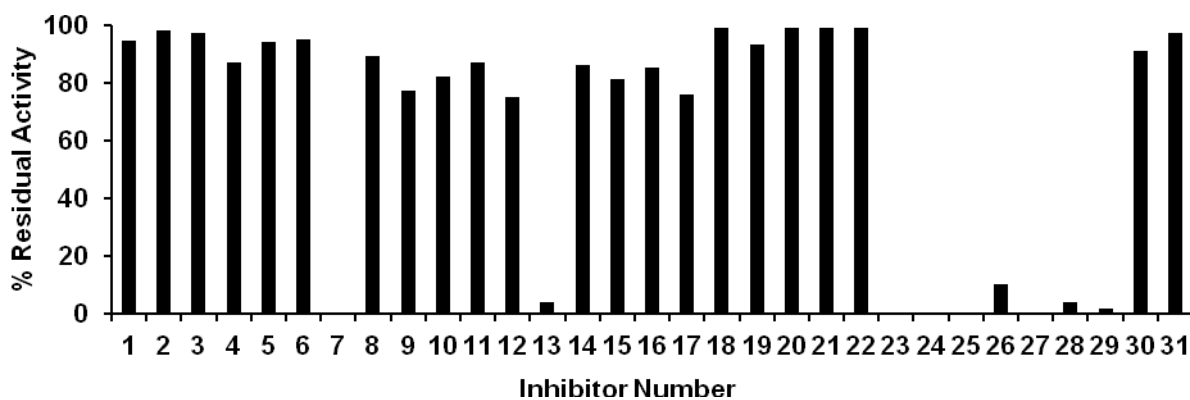
Figure 17: % Residual activity of all the screened molecules of different scaffolds

From **figure 17** it can be seen that compounds **7**, **13**, **23**, **24**, **25**, **27**, **28** and **29** exhibited inhibition against FXIa and had a % residual activity of less than 50%. For further analysis and IC$_{50}$ determination compound **13**, a benzofuran dimer and **24**, a benzofuran trimer were chosen

due to their availability and to serve as a representative of other active molecules of the same scaffold. Compound **13** was chosen instead of **7** because the former had better potency and similarly compound **24** was chosen from the series of trimers as it was the most potent. The information from analyses of these representatives could perhaps serve in understanding the behavior of their respective classes.

2.4 Determination of IC$_{50}$ of compounds 24 and 13

The IC$_{50}$s of compounds **24** and **13** were determined by making serial dilutions of the inhibitors such that each consecutive well would have a concentration which is 5/6th of the previous concentration. Two wells of each row of a 96-well plate were kept as blanks by adding water instead of the inhibitor. The buffer used for these experiments was buffer C and the temperature throughout the experiment was maintained at 37°C. To 85 µL of the buffer, 5 µL of the inhibitor and 5 µL of enzyme (final concentration in each well was 0.765nM) were added. The mixture was then incubated for 10mins followed by addition of 5µL of substrate (final concentration being 345µM). The initial rate of p-nitroaniline formed was determined by monitoring the absorbance at 405nm in the form of the slope of the reaction. The % residual activity for these compounds was determined with respect to the blanks. A plot of the % residual activity versus the log of concentration of the inhibitor was made and fitted using the equation for IC$_{50}$,

$$Y = Y_O + \frac{Y_M - Y_O}{1 + 10^{(\log[I]_O - \log IC_{50})HS}}$$

The inhibition profiles of compounds **13** and **24** are given in **figure 18**,
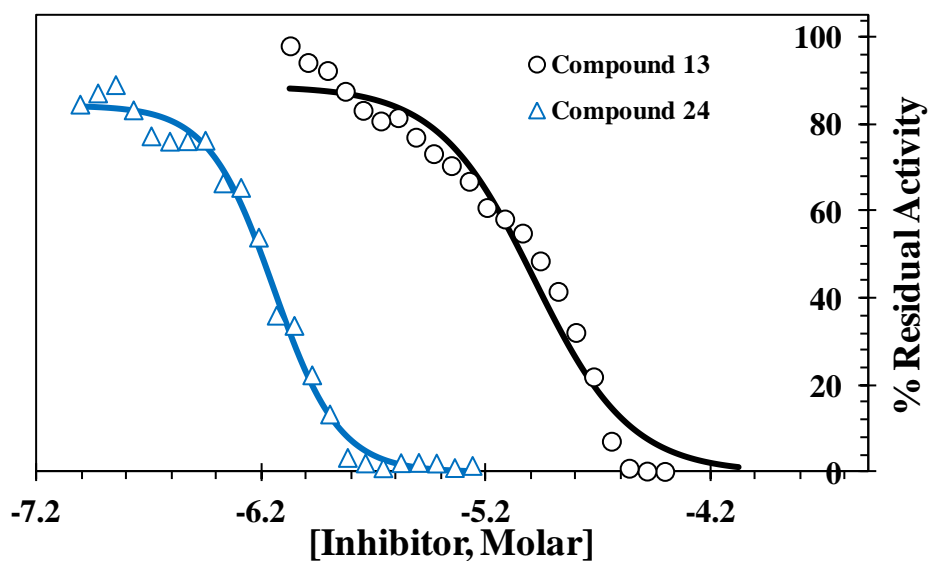
Figure 18: IC$_{50}$ profiles for compounds 13 and 24 against FXIa

The IC$_{50}$s of compounds **13** and **24** are 10.3 ± 0.7 µM and 0.69 ± 0.03 µM.

2.5 Determination of K$_D$ of compounds 13 and 24 for FXIa

The K$_D$ of compounds **13** and **24** were determined by using FXIa labeled with Fluorescein-EGR.

2.5.1 Materials:

Human FXIa tagged with FEGR at the active site was purchased from Haematologic Technologies Inc. (Essex Junction, VT). All other chemicals and reagents were purchased either from Sigma Aldrich (St.Louis, MO) or from Fisher Scientific (Pittsburgh, PA) and were of biochemical grade.

2.5.2 Procedure:

The final concentration of the enzyme FXIa-FEGR in the micro-cuvette was 74 nM. The change in fluorescence of FXIa-FEGR was measured in the presence of increasing concentrations of the inhibitor (Compound **24** or **13**). A temperature of 37°C was maintained throughout the experiment and the slit widths of both emission and excitation were 1mm apart. For the experiment, a semi-micro quartz cuvette was used and with 2 mm and 10 mm path length on the excitation and emission side, respectively, and containing 250 µL total volume was used. The buffer used for this experiment was Buffer C (150mM NaCl, 50mM Tris, 0.1% PEG 8000 at pH = 7.4). The excitation and emission spectra were set at 490nm and 522nm respectively.

2.5.3 Result:
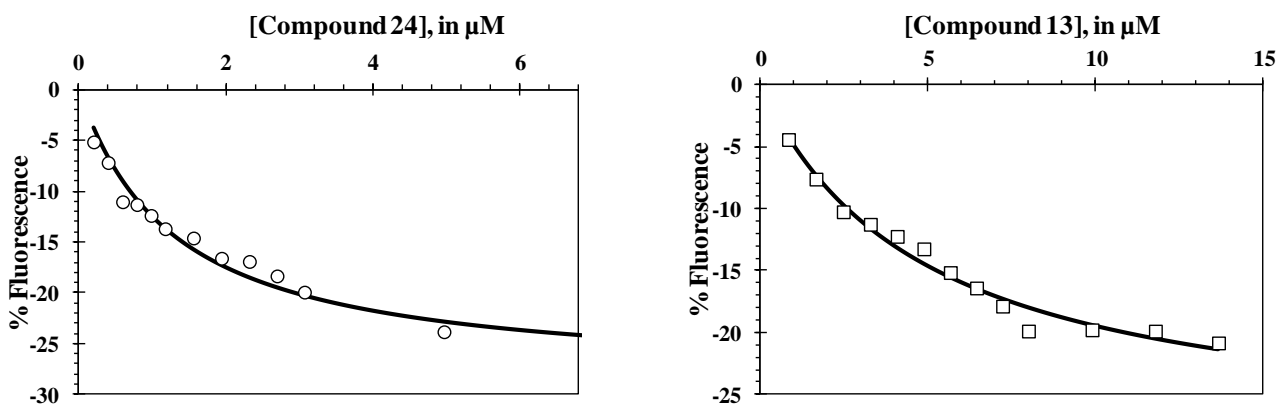
Figure 19: Determination of K_D of compounds 24 and 13 for FXIa-FEGR

The K_D of compounds **24** and **13** (**Figure 19**) for FXIa-FEGR were calculated using SigmaPlot and the equation,

$$\frac{\Delta F}{F_0} = \frac{\Delta F_{MAX}}{F_0} \left\{ \frac{Q - \sqrt{Q^2 - 4[E][I]}}{2[E]} \right\}$$

$$Q = [E] + [I] + K_D$$

The K_D determined for compound **24** and **13** are 1.22 ± 0.16 μM and 4.86 ± 0.62 μM respectively.

2.6 Detecting "promiscuous inhibitors"

A major problem in screening several inhibitors of different scaffolds against a biological target is the development of false positives. Such "inhibitors" bring about inhibition by forming colloidal aggregates, which takes place through "self-association of the organic molecules in the aqueous solution".[61] These aggregates are reported to be hundreds of nanometer in diameter and are reported to sequester the protein thereby giving the false impression that it is inhibiting it. These aggregates are thought to act by either getting absorbed on the surfaces of the enzyme or by enveloping the enzyme within themselves.[61-63]

Such "promiscuous" compounds which form aggregates in solution are highly sensitive to non-ionic detergents. Adding detergent to the assay buffer reduces the aggregability of molecules, which eliminates inhibition. Detergents in the concentrations of 0.01-0.1% cause disruption of aggregates (if formed) and also prevent interaction of aggregates with the enzyme.[64-65] In such a way, a non-ionic detergent differentiates inhibitors from false positives. In order to determine whether compounds **13** and **24** inhibited in a non-specific way, their inhibition profiles were determined in buffers having varying concentrations of the detergent, Tween-80. These profiles were then compared to the profile obtained in the absence of a detergent. Molecules which inhibit an enzyme in the absence of a detergent but not in its presence are most probably undergoing aggregate formation.

2.7 Inhibitory profiles in different concentrations of Tween-80

2.7.1 Materials:

The enzyme human FXIa was purchased from Haematologic Technologies (Essex Junction, VT) and the substrate S-2366 (*L*-pyroglutamyl-*L*-prolyl-*L*-arginine-*p*-nitroaniline) was purchased from Diapharma (West Chester, OH). All the other chemicals were purchased either from Sigma Aldrich (St.Louis, MO) or from Fisher Scientific (Pittsburgh, PA) and were of biochemical grade.

2.7.2 Procedure:

The IC_{50} of compounds **13** and **24** were determined in a similar manner as the one mentioned before. The buffer used for the experiment was buffer C which contained 150 mM NaCl, 50 mM Tris, 0.1% PEG-8000 and 0.02% Tween-80 at pH=7.4. In addition to this buffer, four other similar buffers were prepared differing only in their concentration of Tween-80 (absence, 0.05%, 0.1% and 0.5%). The plots of % residual activity against the log of concentration of the inhibitors are shown in **figures 20 and 21**.
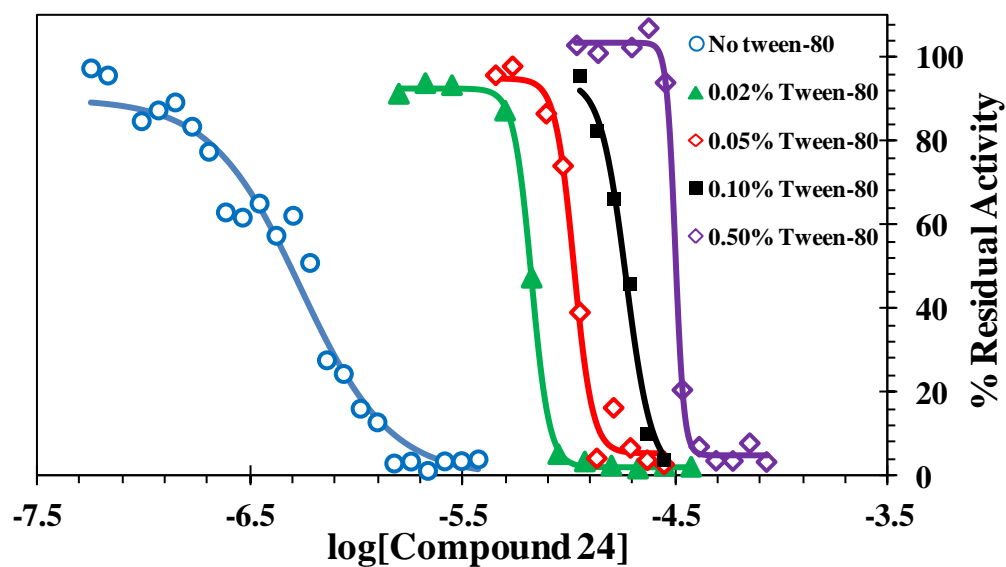
Figure 20: IC$_{50}$ profiles of compound 24 in presence of varying concentrations of Tween-80 in buffer
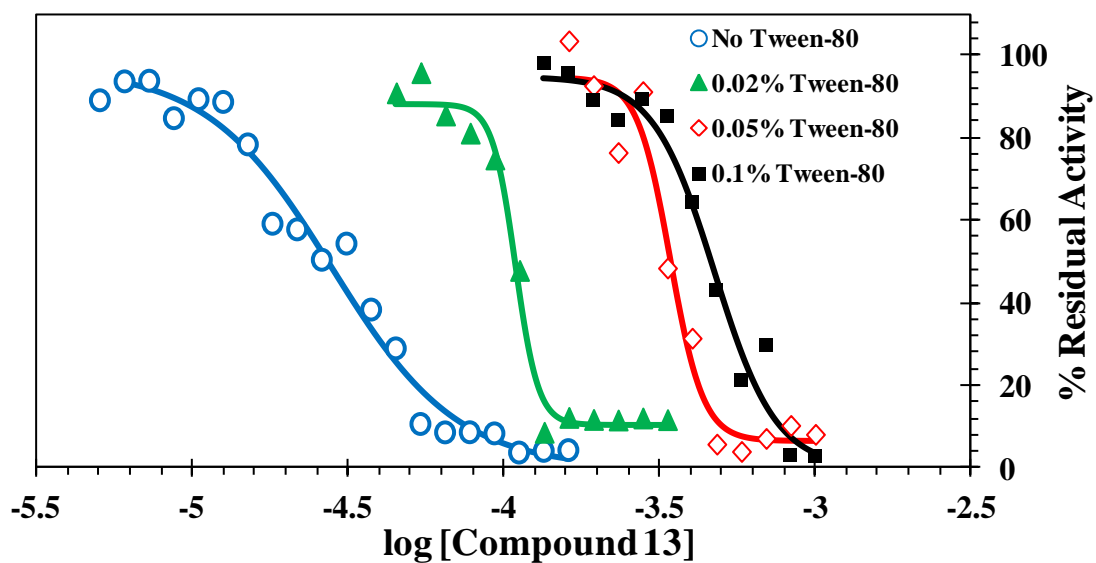
Figure 21: IC$_{50}$ profiles of compound 13 in presence of varying concentrations of Tween-80 in buffer.

2.7.3 Results:

Percentage of Tween-80	Compound 24 (µM)	Compound 13 (µM)
0	0.5 ± 0.04	28.1 ± 2
0.02	6.7 ± 0.03	109.3 ± 2.4
0.05	10.6 ± 0.3	342.3 ± 16
0.1	18.6 ± 1.0	477.2 ± 23
0.5	31.7 ± 0.2	>1mM

Table 7: IC$_{50}$s of compounds 24 and 13 for FXIa in increasing concentrations of Tween-80

It has been reported that any compound which acts as an inhibitor in the absence but not presence of detergent is most probably a promiscuous or aggregate-based inhibitor.[65] Also, the inhibitory action of such compounds is found to be completely destroyed in the presence of a detergent and such a property is characteristic of inhibition by aggregate formation. Upon increasing the detergent concentrations the results obtained **(Table 7)** indicate that although there is a change in the IC$_{50}$ of compound **13** and **24**, the compounds retained their inhibitory action at a Tween-80 concentration as high as 0.1 – 0.5%. The IC$_{50}$ of compound **13** in the absence of Tween-80 in these experiments was 28.11 µM as opposed to the IC$_{50}$ given previously for the same compound (10.34 µM). These compounds are sensitive to moisture and hence there is a possibility that frequent temperature changes (from storage at -20°C to room temperature) for the solid compound might have lead to a compromised inhibitory action against FXIa. However, the detergent studies were performed on the same day and hence on a comparative basis (absence and presence of Tween-80), a gradual increase in IC$_{50}$ upon increase in concentration of Tween-

80 is observed. A new batch of compound **13** was synthesized and the IC_{50} recorded for this new batch was similar to the old batch (~10 µM).

Detergents at low concentrations are said to activate certain enzymes but when present in high concentrations are found to reduce enzyme activity. It has also been reported that high concentrations of detergents are found to reduce the inhibitory activities of certain inhibitors. This loss in activity is attributed to the formation of detergent micelles.[66] These also could be the reasons for the increase in IC_{50} observed. Apart from this, the inhibitory activity of these compounds did not completely disappear even in the presence of very high concentrations of Tween-80. As a result, the possibility of inhibition by non-specific or promiscuous means was ruled out.

Chapter 3: Biophysical Characterization

3.1 Determining the type of inhibition by Michaelis-Menten kinetics

Although FXIa was found to be inhibited by compounds **13** and **24**, whether inhibition was due to binding to active site of the enzyme or an allosteric site was not known. A means of determining this would be to compare the V_{max} and K_m of the FXIa-catalyzed substrate hydrolysis as a function of substrate concentrations for different concentrations of inhibitor. These values were then compared to the V_{max} and K_m in the absence of the inhibitor.

3.1.1 Materials:

Human Factor XIa was purchased from Haematologic Technologies Inc. (Essex Junction, VT). The chromogenic substrate *L*-pyroglutamyl-*L*-prolyl-*L*-arginyl-*p*-nitroaniline (S-2366) was purchased from DiaPharma (West Chester, OH). All the other materials or chemicals were of biochemical grade and purchased either from Sigma Aldrich (St.Louis, MO) or from Fisher Scientific (Pittsburgh, PA).

3.1.2 Procedure:

The buffer used for these experiments consisted of 150mM NaCl, 50mM Tris, 0.1% PEG 8000 at a pH = 7.4 and the temperature throughout the experiment was maintained at 37°C. For compound **13**, three different concentrations (10 µM, 5 µM and 7.25 µM) and similarly for compound **24** four different concentrations were used (150 nM, 300 nM, 750 nM and 900 nM). The initial rate of substrate hydrolysis by FXIa was measured at 405nm using the microplate reader, FlexStation III (Molecular Devices). The K_m and V_{max} values obtained for each

concentration of the inhibitors is given in **tables 8** and **9**. The rate of substrate hydrolysis by the

enzyme in the absence of the inhibitors was also determined. (**Figure 22** and **23**)
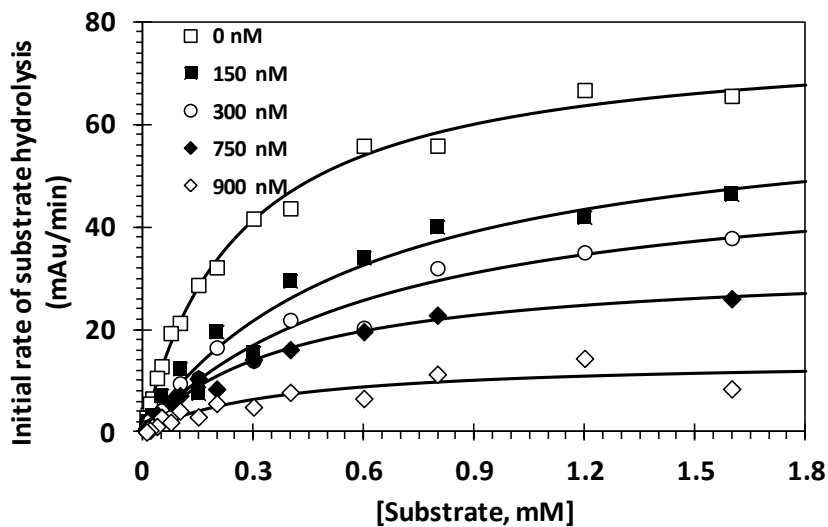
Figure 22: Michaelis-Menten kinetics of compound 24
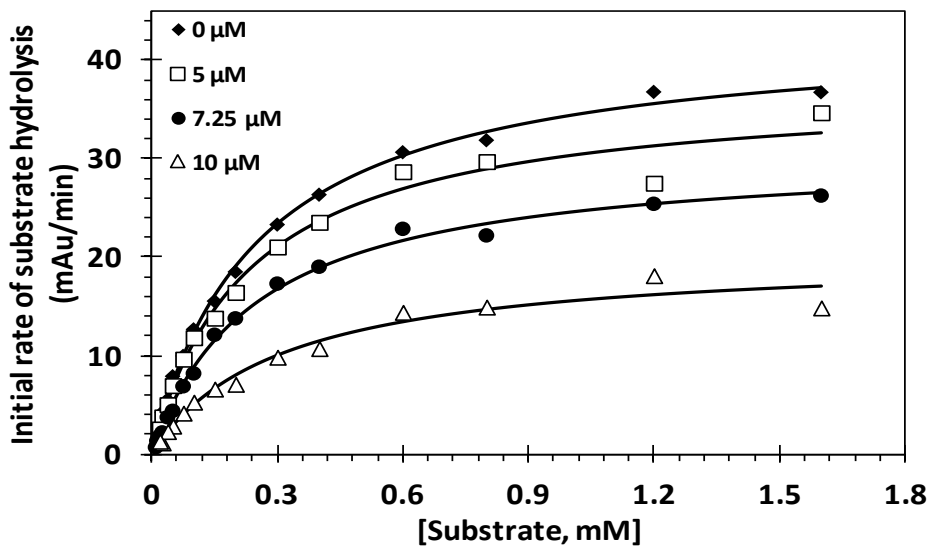
Figure 23: Michaelis-Menten kinetics of compound 13

3.1.3 Conclusions drawn from Michaelis-Menten kinetics

The values of K_m and V_{max} were obtained by fitting the data obtained to the Michaelis-Menten equation and are given in **tables 8** and **9**.

$$V = \frac{V_{max}\,[S]}{[S] + K_m}$$

[Compound 24]	V_{max}	K_m (mM)
0 nM	77.4 ± 1.7	0.26 ± 0.02
150 nM	65.2 ± 6.3	0.60 ±0.13
300 nM	53 ± 5	0.64 ± 0.13
750 nM	45.5 ± 5.6	0.64 ± 0.04
900 nM	14.2 ± 2.2	0.38 ± 0.15

Table 8: K_m and V_{max} at different concentrations of compound 24

[Compound 13]	V_{max}	K_m (mM)
0 µM	43.1 ± 0.6	0.25 ± 0.01
5 µM	37.4 ± 1.4	0.23 ± 0.02
7.25 µM	30.7 ± 0.6	0.25 ± 0.01
10 µM	20.3 ± 1.2	0.31 ± 0.04

Table 9: K_m and V_{max} at different concentrations of compound 13

At different concentrations of both **24** and **13**, it can be seen that there is a change in the V_{max} (decrease with increasing inhibitor concentration) of the FXIa-catalyzed substrate hydrolysis while there seems to be no significant change in the K_m. Such observations in the K_m and V_{max} are a characteristic of non-competitive inhibition. Hence, from these results it can be concluded that **13** and **24** are inhibiting FXIa by binding to a site other than the active site. In order words, they are inhibiting FXIa by binding to an allosteric site.

3.2 Fluorescence spectroscopy for compounds 13 and 24 with FXIa-FEGR

The Michaelis-Menten kinetics study showed that the compounds **13** and **24** were perhaps inhibiting FXIa through an allosteric mechanism. To further assess whether inhibitor **24** and **13** were indeed binding at a site other than the active site, we resorted to fluorescence-based detection of conformational change in the active site. A fluorophore, Fluorescein-EGR (FEGR) - labeled at the active site of FXIa was purchased from Haematologic Technologies. Fluorescein **(Figure 24)** is a well-established fluorophore that is particularly sensitive to changes in electrostatics of the area adjacent to its location and serves as an excellent probe for conformational changes upon ligand binding.
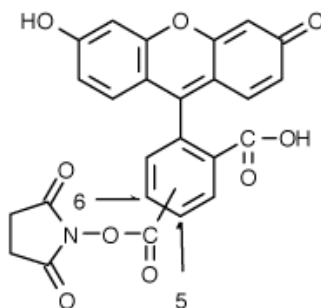
Figure 24: Structure of succinimidyl ester linked fluorescein

Since the inhibitors are proposed to bind at an allosteric site, the FEGR label was introduced at the active site of the enzyme by reacting FXIa with Fluorescein-Glu-Gly-Arg-chloromethylketone (FEGRck). Active serine proteases are usually tagged with a fluorescent probe by incubating the protease with FEGRck for 2hrs at room temperature.[67,68] The protein aliquots are then analyzed using SDS-PAGE. When FXIa is treated with FEGRck, the methylene group reacts with the active site histidine residue which displaces the chloride and ultimately the enzyme is tagged by formation of a covalent bond.[67] By tagging the enzyme in such a manner, its catalytic activity is inhibited and it is possible to study the molecular changes that might be brought about at the active site due to allosteric inhibitors. If the binding of the compound to the allosteric site is bringing about a conformational change in the active site of the enzyme then there would be a change in the fluorescence (quantum yield or λ_{EM}) of FEGR.

The excitation and emission wavelength of FEGR is 490 and 520 nm respectively. In fluorescence experiments there is a possibility of interference in the measurements due to other components that are present in the mixture. This can interfere with the actual changes in fluorescence of FEGR giving rise to inaccurate results which are undesirable. In order to eliminate the possibility of interference caused by the inhibitors, their absorption spectra were determined.

3.2.1 Materials

The human FXIa labeled with FEGR, referred to as FXIa-FEGR, was purchased from Haematologic Technologies (Essex Junction, VT). The chromogenic substrate *L*-pyroglutamyl-*L*-prolyl-*L*-arginyl-*p*-nitroaniline (S-2366) was purchased from DiaPharma (West Chester, OH).

All the other materials or chemicals were of biochemical grade and purchased either from Sigma Aldrich (St.Louis, MO) or from Fisher Scientific (Pittsburgh, PA). The absorption spectra were recorded by using a Shimadzu UV/Vis Spectrophotometer using a semi-micro quartz cuvette and the buffer used was Buffer C.

3.2.2 Effect of compound 24 binding to FXIa-FEGR

For compound **24** the absorption spectrum **(Figure 25)** was recorded prior to the fluorescence experiments.
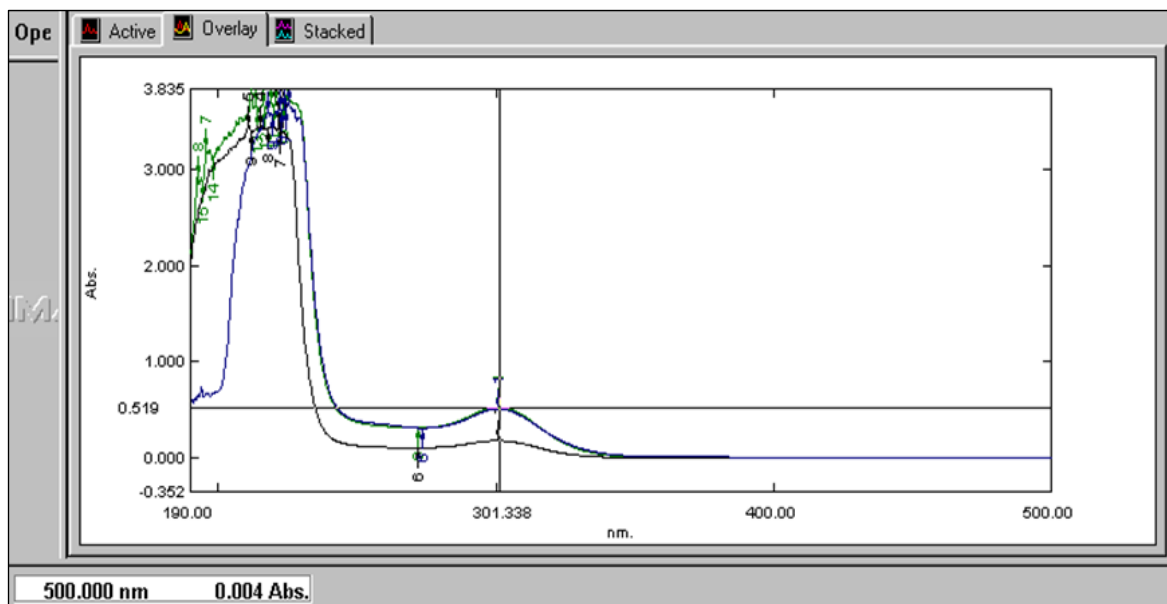
Figure 25: Absorption spectra for compound 24

The fluorescence emission spectra of FXIa-FEGR and appropriate controls were measured on a Photon Technology International (Birmingham, NJ) spectrofluorometer at a constant temperature of 37°C with slit width for excitation and emission channels set to 1 mm. The concentrations of FXIa-FEGR and compound **24** were 73.8 nM and 8 µM, respectively. A

semi-micro quartz cuvette with 2 mm and 10 mm path length on the excitation and emission side, respectively, and containing 250 µL total volume was used. A Tris-HCl buffer, pH 7.4, containing 50 mM Tris, 150 mM NaCl, and 0.1% PEG 8000 was used for measurements. The excitation wavelength was set at 480 nm and the emission spectrum was collected from 500 – 600 nm (**Figure 26**).
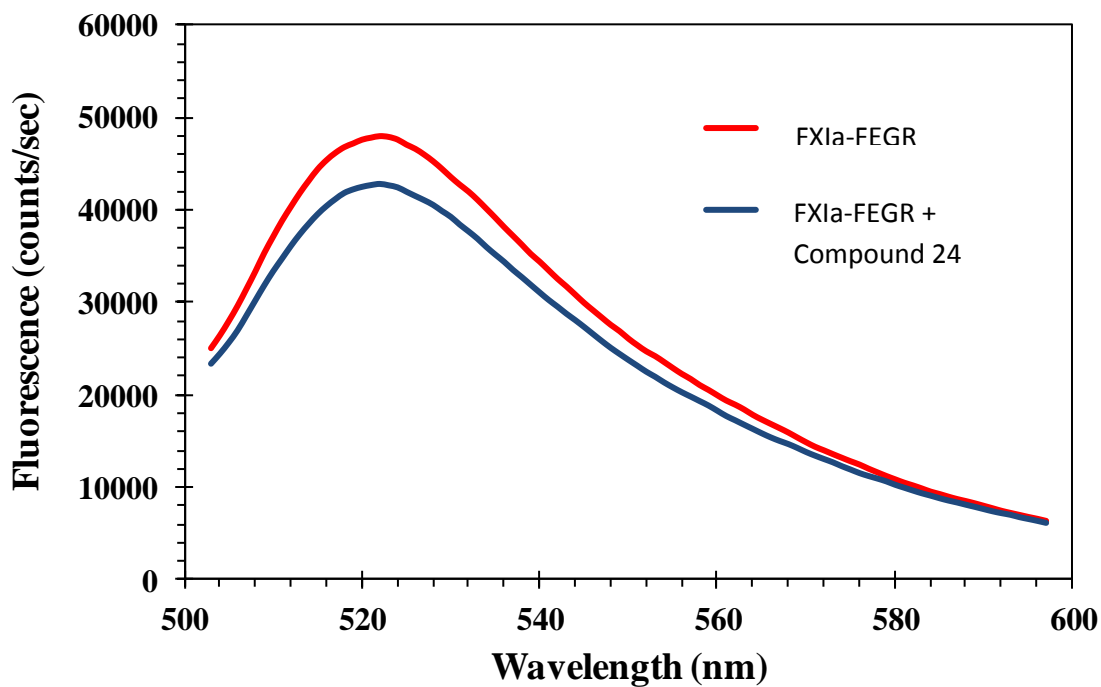
Figure 26: Emission scan of FXIa-FEGR in presence and absence of compound 24

The absorption spectrum (**Figure 25**) of **24** shows that the compound does not absorb at either the excitation (490 nm) or the emission wavelength (520 nm) of the fluorophore FEGR used for these experiments. Hence the possibility that there could be interference in the fluorescence measurements by the inhibitor can be eliminated. Similar results were observed for compound **13**.

The emission scan **(Figure 26)** shows that in the presence of compound **24** the fluorescence of FXIa-FEGR is reduced in comparison to FXIa-FEGR alone. At the λ_{MAX} (522 nm), presence of 8 μM compound **24**, reduces the counts from 48,042 to 42,756 indicating a loss of ~11% in fluorescence intensity. This loss is substantial and suggestive of an inhibitor induced alteration in the electrostatics of the active site geometry, which contains the fluorophore. A plausible explanation for this decrease in fluorescence intensity is compound **24** induced a conformational change in the enzyme's active site. This also implies that inhibitor **24** binds at a site remote from the active site to induce an allosteric conformational change.

3.2.3 Effect of compound 13 binding to FXIa-FEGR

The absorption spectrum for **13** was recorded in a similar manner as that mentioned for **24** and is given in **figure 27**. The buffer used was buffer C.

Also, the emission scan of FXIa-FEGR was measured in the absence and presence of **13** **(Figure 28)**. The concentrations of **13** and FXIa-FEGR were 120 μM and 74 nM, respectively. The slit width for both excitation and emission was set at 1mm and a temperature of 37°C was maintained throughout the experiment. The emission scan shows a drastic decrease in the fluorescence counts of FXIa-FEGR in presence of **13** as compared to the fluorescence in its absence. This effect seems to be similar to the one seen with compound **24**. However, when the inhibitor was added gradually in the semi-micro cuvette, a rise in the fluorescence was observed with increasing concentration. When the emission scan (ranging from 490-520 nm) of **13** was recorded using the PTI Spectrofluorometer, the compound did not exhibit any fluorescence. It is quite possible that the compound exhibited fluorescence upon binding to the enzyme.
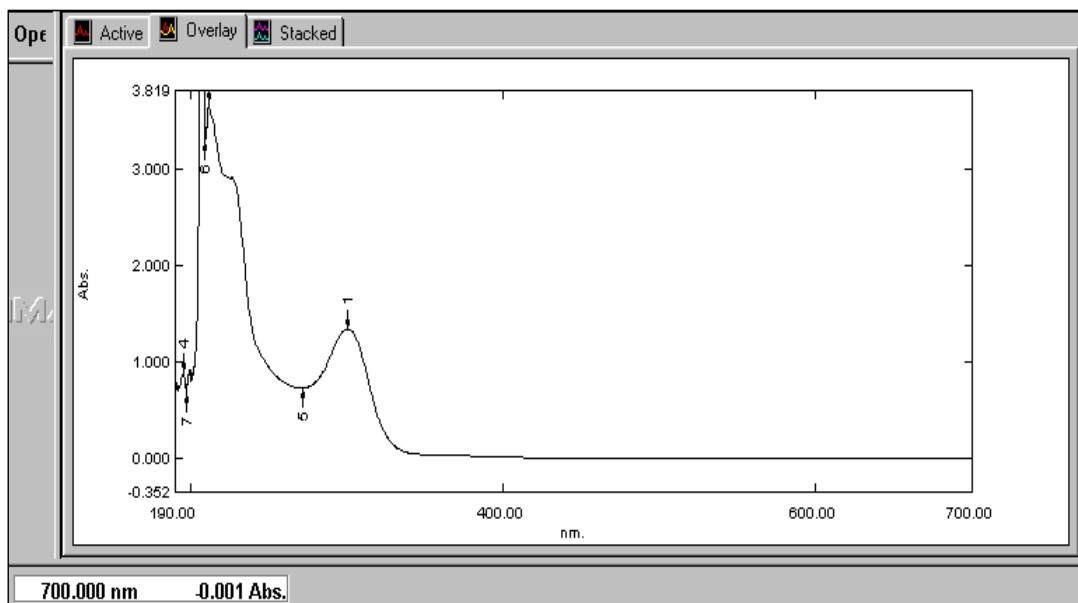
Figure 27: Absorption spectrum for compound 13
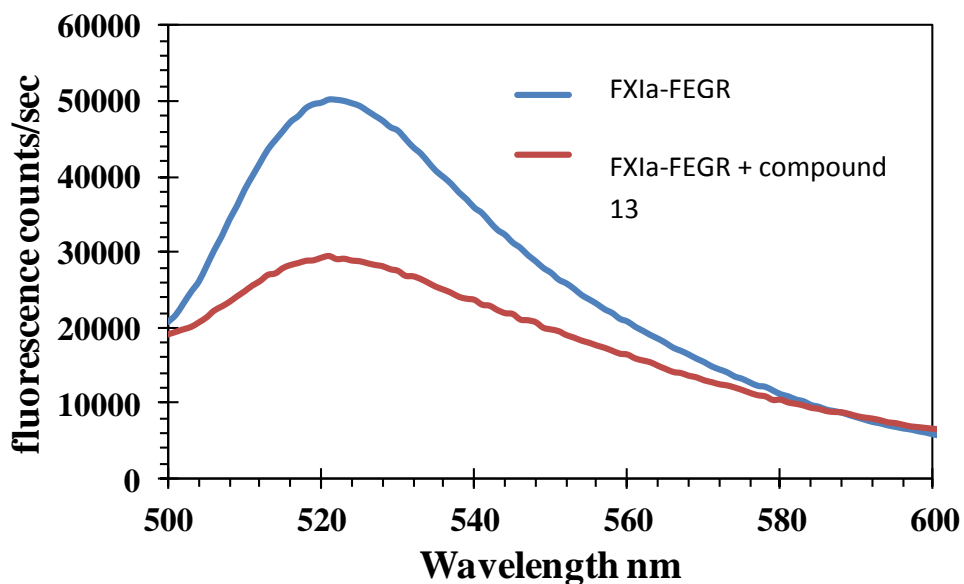
Figure 28: Emission scan of Compound 13 (~120 μM)

In the emission scan **(Figure 28)**, it can be seen that the presence of the inhibitor **13** causes a decrease in the fluorescence of FXIa-FEGR (λ_{MAX} = 523nm) from 35001 counts to 18957 counts, which is ~46% decrease. Since a high concentration of inhibitor could be

contributing to the fluorescence change, the concentration for the quenching experiment was reduced to 40 μM.

3.3 Further studies to confirm allosteric inhibition

To further assess the nature of the allosteric conformational change in the enzyme, fluorescence quenching studies were performed using collisional quenchers, acrylamide and sodium iodide. Fluorescence quenching studies attempt to identify differences in the response of two species, e.g., a fluorophore-labeled protein-ligand complex and a fluorophore-labeled protein alone, to collisional quenchers. If a conformational change in the protein is brought about by the presence of a ligand, the collisional quencher will sense an altered orientation of the fluorophore between the two species. Fluorescence quenching requires the quencher to achieve molecular contact with the excited state of fluorophore. Upon a successful contact, the fluorophore returns to the ground state without releasing a photon.[69] Thus, these studies can differentiate the accessibility of the fluorophore to the quencher in ligand bound and unbound states.

Fluorescence quenching has been utilized a number of times to monitor the changes that occur in protein conformation which could occur due to their environment or components of the environment (such as inhibitors) with which they interact.[69-71] Most of the studies involve quenching of tryptophan residue(s) in single or multiple-tryptophan containing proteins and the quenchers used frequently are acrylamide and iodide.[72] Acrylamide is a polar uncharged molecule which can enter the interiors of the protein and hence can quench fluorescence of hidden Trp residues which are otherwise inaccessible to a larger charged species such as iodide. Fractions of bovine lens crystallins, trypsin, RNase-T 1 and cytochrome c are some of the

proteins that have been studied using quenching.[72-74] Other quenchers used are succinimide, oxygen, pyridine and hydrogen peroxide. The fluorophore, fluorescein tagged to the active site of proteins such as trypsin has been used for quenching experiments especially with iodide being used as a quencher.[75] Iodide quenching studies using fluorescein have also been employed to understand the disposition of cobra α-toxin when it was bound to the surface of acetylcholine receptor as compared to when it was unbound. Here the fluorescein was tagged to a Lys[23] residue of the toxin.[76] Apart from this there are other proteins that have utilized the combination of fluorescein with iodide.[77-79] In 2004, Sinha et al. reported an acrylamide quenching study with DEGR-FXIa in which a conformation change upon binding to certain polyanions was observed. In the presence of a polyanion such as dextran sulfate ($M_r \sim 10000$; DX10) there was an increase in the fluorescence (λ_{MAX}) of DEGR-FXIa. Due to the possibility of interaction of iodide with the highly charged polyanionic molecules, acrylamide was chosen as the quencher. Quenching determined that the DEGR-FXIa bound to DX10 was more resistant to quenching than unbound DEGR-FXIa. This resistance was attributed to a conformation change that may have been brought about in DEGR-FXIa when bound to DX10.[80]

3.4 Quenching studies

All the chemicals and materials purchased are same as mentioned previously.

3.4.1 Fluorescence quenching of FXIa-FEGR in presence and absence of compound 24

The concentrations of FXIa-FEGR and compound **24** were 74 nM and 8 µM, respectively, while the quenchers, acryamide and sodium iodide, were studied at multiple concentrations. The fluorescence measurements were carried out at a constant temperature of 37°C using a quartz cuvette (excitation and emission path lengths of 2 mm and 10 mm, respectively) and with excitation and emission wavelengths set to 480 and 520 nm, respectively. The excitation and emission slit widths were set to 1 mm and 50 mM Tris-HCl buffer, pH 7.4, was used as described above.

Results and conclusions for compound 24

Gradual increase in the concentration of acrylamide from 0 to 0.4 M did not alter the emission of FXIa-FEGR alone (**Figure 29**).
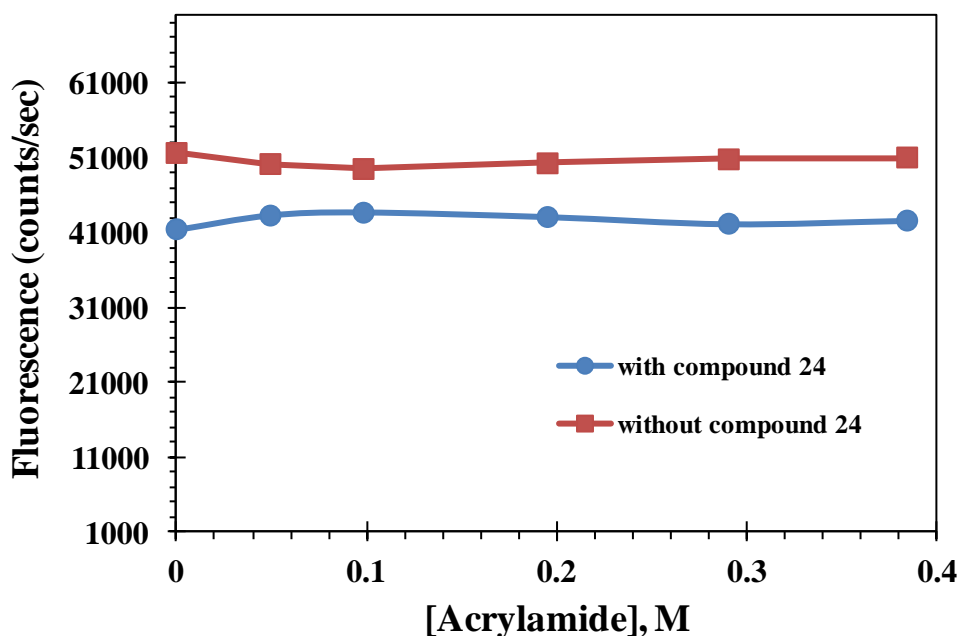
Figure 29: Fluorescence quenching of FXIa with acrylamide in presence and absence of Compound 24

In the presence of 8 μM compound **24**, the fluorescence at 520 nm decreases nearly 11%, as expected (see above). However, the presence of acrylamide (0 → 4 M) does not change this fluorescence appreciably.

The absence of any effect of acrylamide is surprising and interesting. Acrylamide is a small hydrophobic molecule that most probably interacts with inner hydrophobic patches present on FXIa. It is also a dynamic quencher that finds difficulty contacting with the large fluorophore, FEGR, because it's long-range quenching is limited.[79] Thus, it is possible that a larger collisional quencher may be a better choice to identify subtle conformational changes brought about in FXIa.[81]

Another collisional quencher used in the literature is iodide ion as previously mentioned and we studied its ability to quench the FXIa-FEGR fluorescence. **Figure 30** shows the profile of

fluorescence emission intensity in the presence of 0 to 0.175 M sodium iodide. Iodide ion is able to induce quenching in the fluorescence of both species, FXIa-FEGR alone and its complex with compound **24**. Interestingly, the iodide concentration dependence for the species is significantly different suggesting that collisions between the quencher and the fluorophore are significantly different. Alternatively, the FXIa-FEGR bound to the inhibitor is more resistant to quenching than in the absence of the inhibitor. Also, when additional inhibitor **24** (~33 µM) is added to FXIa-FEGR quenched by iodide, the enzyme partially recovers its fluorescence. This supports the hypothesis that compound **24** is able to induce an allosteric conformational change in the active site of FXIa, which may be the reason for inhibition of its catalytic activity. Also, when a saturating concentration of the inhibitor is added to the free FXIa-FEGR quenched by sodium iodide, the enzyme seems to somewhat regain its fluorescence.
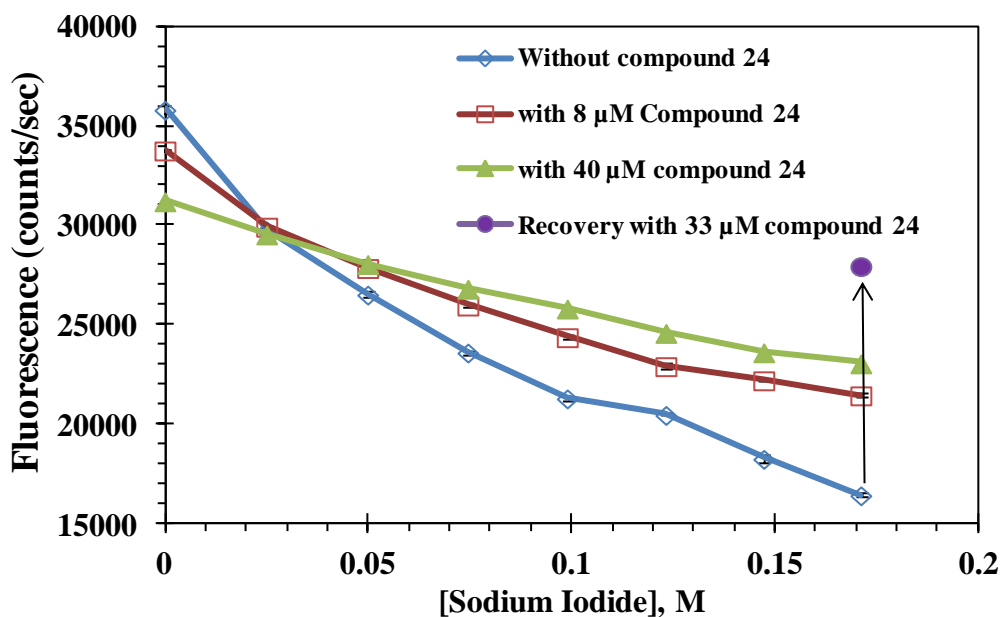
Figure 30: Changes and recovery of fluorescence in FXIa-FEGR by gradual addition of NaI in presence and absence of compound 24

55

Collisional quenching of fluorescence can be described by the Stern-Volmer equation 1,

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D[Q] \ldots\ldots(1)$$

In this, F_0 and F are the fluorescence intensities of the fluorophore in the absence and presence of the quencher, k_q is the bimolecular quenching constant, τ_0 is the fluorophore lifetime in the absence of the quencher and Q is the concentration of the quencher. In this equation, K_D, equal to $k_q \tau_0$, is the Stern-Volmer constant for dynamic quenching (not equilibrium dissociation constant).[14] The modified Stern-Volmer equation, which is useful when the change in fluorescence is very small at low quencher concentrations is given by,

$$\frac{F_0}{\Delta F} = \frac{1}{K_D[Q]} + 1$$

A plot of $\frac{F_0}{\Delta F}$ versus $\frac{1}{[Q]}$ for the enzyme both, in presence and absence of the inhibitor, is called the Stern-Volmer plot.[69,80]
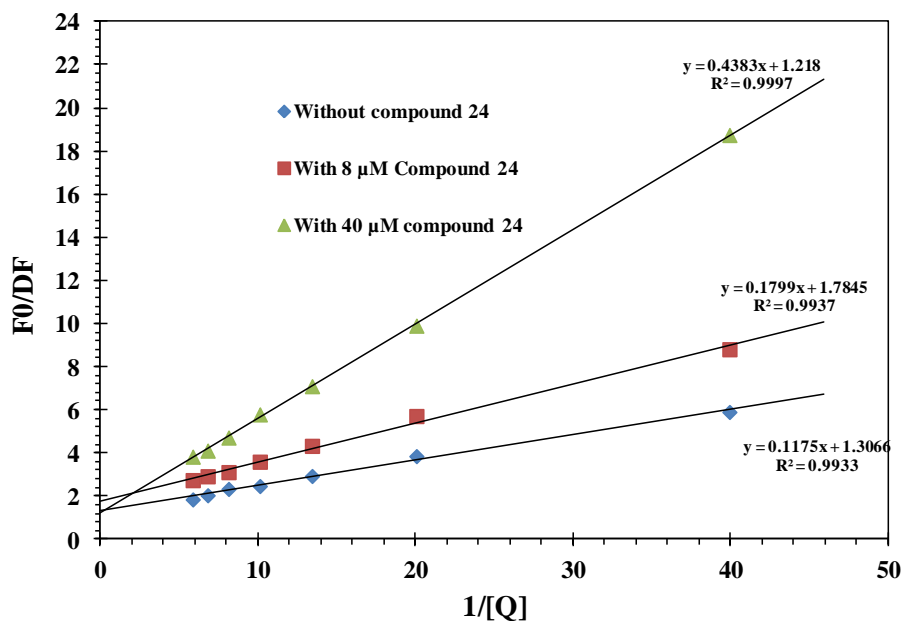
Figure 31: Stern-Volmer plot for FXIa-FEGR in absence and presence of compound 24 (8 and 40 μM)

A characteristic feature of the Stern-Volmer plot is that assuming that the solution consists of only one species, the y-intercept of the plot will always be 1.0.[80] As can be seen from **Figure 31**, the Stern-Volmer plot for FXIa—FEGR alone intersects the y-axis at 1.0. Likewise, the plot in the presence of a saturating concentration of 40 μM compound **24** also intersects at 1.0. However, in the presence of 8 μM inhibitor, the intersect is ~1.8, which indicates the presence of two different species of fluorophores, free and bound FXIa-FEGR.

In conclusion, the resistance of compound **24** – FXIa to quenching by sodium iodide in comparison to FXIa alone could be due to a conformational change in the active site brought about by binding of the inhibitor. The induced conformational change most probably makes the fluorophore less accessible to the collisional quencher. Alternatively, the binding to the inhibitor

to FXIa results in a steric clash with the incoming iodide. This could arise from the inhibitor binding at a site near the active site that bears the FEGR label.

3.4.2 Fluorescence quenching of FXIa-FEGR in absence and presence of compound 13.

The quenching studies on compound **13** can be seen in **figures 32** and **33.**
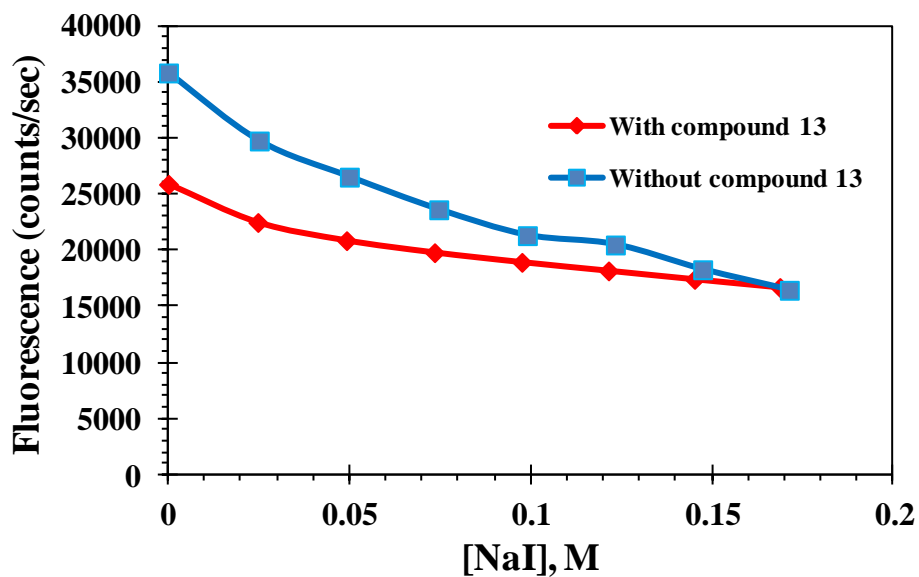
Figure 32: Quenching of fluorescence by NaI in presence and absence of compound 13
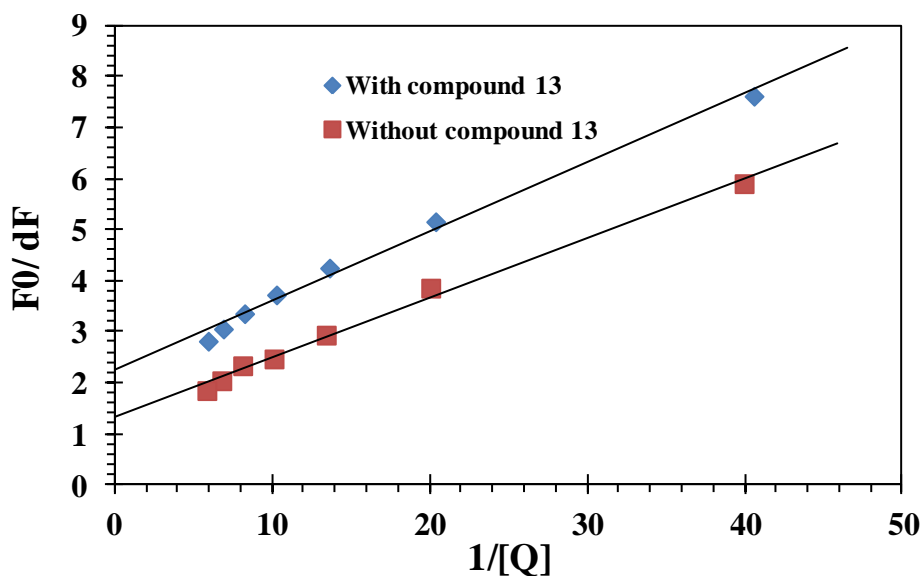
Figure 23: Stern-Volmer plot for FXIa-FEGR in presence and absence of compound 13

The Stern-Volmer plot **(Figure 33)** shows that the plot in the presence of inhibitor **13** (40 µM) intersects at ~2.2. This shows that there was more than 1 species of fluorophore present in the solution which could possibly be free FXIa-FEGR and compound **13** bound FXIa-FEGR. Perhaps the concentration of the inhibitor (40 µM) was not sufficient to saturate all the binding sites and hence the intercept on the y-axis is higher.

Results and conclusions for compound 13

The Michaelis-Menten studies show that compound **13** is inhibiting FXIa by an allosteric mechanism. However, for the quenching experiments, the possibility of interference in fluorescence measurements due to the inhibitor has to be accounted for. Further investigation perhaps will help in achieving clarity regarding how binding of the inhibitor to FXIa could cause a conformational change in the active site of the enzyme.

Chapter 4: Competition with heparin

4.1 Interaction of heparin with FXIa

As the coagulation cascade enzymes prepare to form a stable clot upon injury, there is another set of interactions of certain proteins that intend to stop clot formation and therefore are anti-coagulant in nature. One such natural anti-coagulant is antithrombin (AT) which binds and inhibits thrombin. AT has a low affinity for thrombin however in the presence of heparin, a highly sulfated glycosaminoglycan (GAG), AT shows a very high affinity for thrombin. Apart from thrombin, heparin is reported to indirectly inhibit FXa, FXIIa and FXIa as the inhibition of these enzymes is dependent upon the presence of AT.[82,83]

The regulation of FXIa is known to be carried out by several serine protease inhibitors or serpins. Earlier findings had reported that α_1-antitrypsin as the major inhibitor of FXIa with antithrombin playing a significant role. However, recent findings have shown that in fact C1-inhibitor plays an important role in regulating FXIa while α_2-antiplasmin is said to make an essential contribution.[84,85] Another serpin, AT is also found to inhibit FXIa and this inhibition is found to be potentiated in the presence of heparin amongst other GAGs.[86] The mechanism of potentiation of inhibition is carried out when both FXIa and AT bind to the same molecule of heparin. AT is also known to undergo conformational changes upon binding to heparin which further contribute to potentiate inhibition.[26,88] Heparin is also said to potentiate the inhibition of FXIa by the kunitz-type protease inhibitor, PNII in which both, FXIa and PNII bind to the same heparin molecule.[87,89] Since heparin is reported to be involved not only in activation but also in inhibition of FXIa, there has to be an interaction between these two species. Heparin is known to

bind to the apple-3 domain of FIX bearing the heparin-binding consensus sequence, [250]R-I-K-K-S-K[255]. Mutagenesis studies by Zhao et al. involved replacement of basic amino acid residues, Lys[252], Lys[253] and Lys[255] by Ala. By replacing Lys[253] with Ala[253] it was seen that inhibition of FXIa by AT reduced 4-fold compared to wild-type FXIa and smaller reductions were observed when residues Lys[252] and Lys[255] were replaced by Ala.[84] Not only inhibition but also autoactivation was affected (reduced) more significantly when Lys[253] was replaced with Ala and to a lesser extent when Lys[252] and Lys[255] were replaced with Ala. These studies showed that Lys[253] is the most important amino acid required for binding to heparin while Lys[252] and Lys[255] are also residues considered important for interactions with heparin. However, if this were the only heparin-binding site on FXIa then substitution of the lysine residues with Ala should result in a mutant which is incapable of binding to heparin. A reduction but not a complete loss in heparin-binding property of this mutant indicates the possibility of another heparin-binding site on FXIa.[84,90]

Following the above mentioned observation, the primary amino acid sequence of FXIa was scanned to detect if any of the amino acid sequences were homologous to the known heparin-binding sequences found in other proteins. Usually heparin binding sites are found to be rich in arginine and lysine amino acids and are commonly separated by one hydrophobic amino acid. Apart from this, amino acids leucine, tyrosine and tryptophan are also found commonly in the heparin-binding site. When FXIa was scanned for amino acid sequences homologous to the sequences to which heparin binds, the two residues that were found were, [509]YRKLRDK[515] and [527]CQKRYRGHKITHKMIC[542] located in the catalytic domain. The latter sequence is a carboxyterminal cysteine-constrained loop which is observed in other heparin-binding enzymes

such as FIXa and thrombin. In fact, the arginine residues in this sequence in thrombin makes up a part of exosite 2, which is a known heparin binding site. Results of certain studies suggest that residues Lys^{529}, Arg^{530} and Arg^{532} in the cysteine-constrained loop is important for binding of FXIa to GAGs.[87]

The important residues involved in the interaction with heparin are shown in **figure 34** in the color cyan. (The software used for generating this image was The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. PDB ID 2F83)
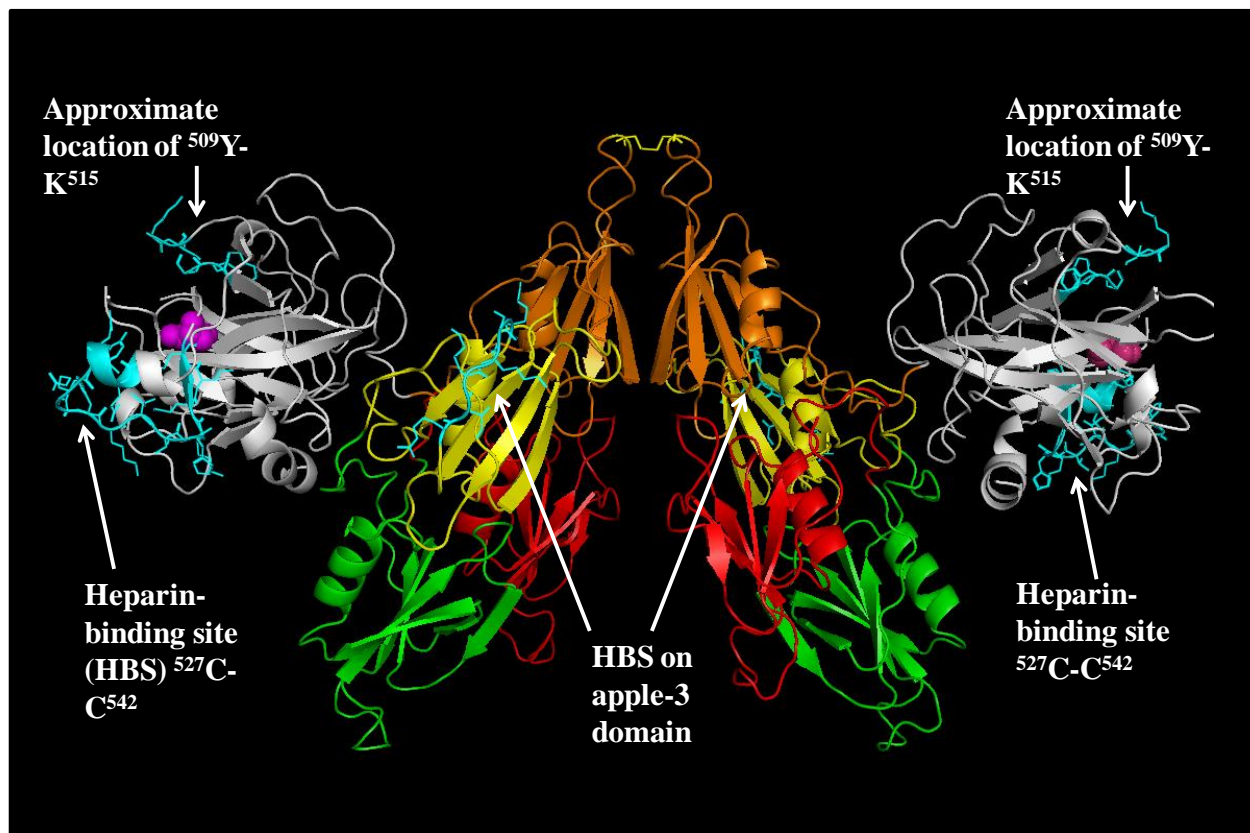
Figure 34: Important heparin-binding sites on the catalytic domain and apple-3 domain of FXI

Compounds **13** and **24** are sulfated small molecule inhibitors which are designed to act as GAG mimetics. The binding site of these small inhibitors on FXIa was not known and hence it was interesting to determine whether these molecules were binding at the same site as that of heparin. In order to do so, the potency of these molecules for FXIa was determined in the presence of 3 different concentrations of heparin-Na$^+$. Prior to these experiments the potency of heparin-Na$^+$ against FXIa only was determined.

4.2 Determination of K_D and IC_{50} of heparin for FXIa

4.2.1 Materials:

Human coagulation factor XIa was purchased from Haematologic Technologies Inc (Essex Junction, VT) and the substrate S-2366 (*L*-pyroglutamyl-*L*-prolyl-*L*-arginine-*p*-nitroaniline) was purchased from Diapharma (West Chester, OH). Heparin-Na$^+$ (Acros Organics) and all other chemicals were purchased either from Sigma Aldrich (St.Louis, MO) or from Fisher Scientific (Pittsburgh, PA) and were of biochemical grade.

4.2.2 IC_{50} determination:

The IC_{50} was measured by making serial dilutions of heparin-Na$^+$ such that each dilution was 1/10th of the previous. Another series of dilutions was prepared which were 1/3rd dilutions of the first series. The final concentration of the enzyme FXIa was 1.53 nM while that of substrate was 345 μM and the buffer used was Buffer C (previously mentioned). In each well, 85 μL of Buffer C (temperature maintained at 37°C) was added followed by 5 μL of heparin-Na$^+$ and 10 μL of FXIa. The mixture was allowed to incubate at 37°C for 10mins after which 5 μL of substrate was added and the initial rate of substrate hydrolysis by FXIa was immediately

recorded at 405nm using FlexStationIII (Molecular Devices, Sunnyvale, CA). The IC_{50} curve

(**figure 35**) was plotted using the previously mentioned equation,

$$Y = Y_O + \frac{Y_M - Y_O}{1 + 10^{(\log[I]_O - \log IC_{50})HS}}$$
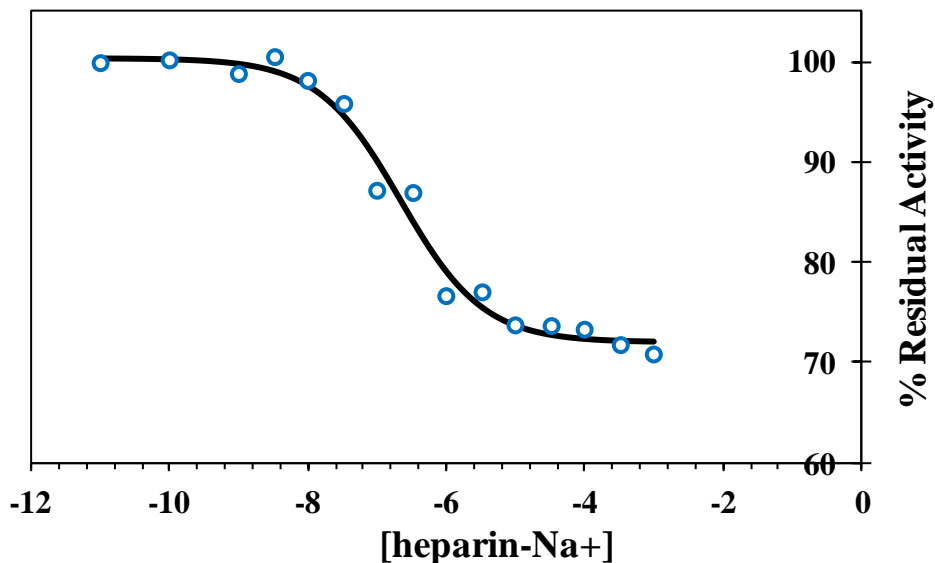
Figure 35: IC_{50} profile of heparin-Na$^+$ for FXIa

4.2.3 Determination of K_D

The K_D of heparin for FXIa was previously determined in our lab (The Desai Lab) by measuring the changes in the intrinsic fluorescence of FXIa. The enzyme and other materials or chemicals were purchased from the same sources as those previously mentioned. The experiment was carried out at a constant temperature of 37°C and the buffer used consisted of 20mM NaPi, 100mM NaCl, 0.1% PEG-8000, 0.1mM EDTA at a pH of 7.4. The excitation and emission wavelength were set at 280 and 340nm respectively. The slit width of both excitation and

emission were set at 1mm. A stock concentration of 200 μM was prepared in water and the change in intrinsic fluorescence of FXIa was observed when this solution was gradually added. The final concentration of FXIa was ~ 22 nM. A plot of % fluorescence against the concentration of heparin is shown in **figure 36**.

The K_D of heparin for FXIa was found to be 1.3 ± 0.003 μM.
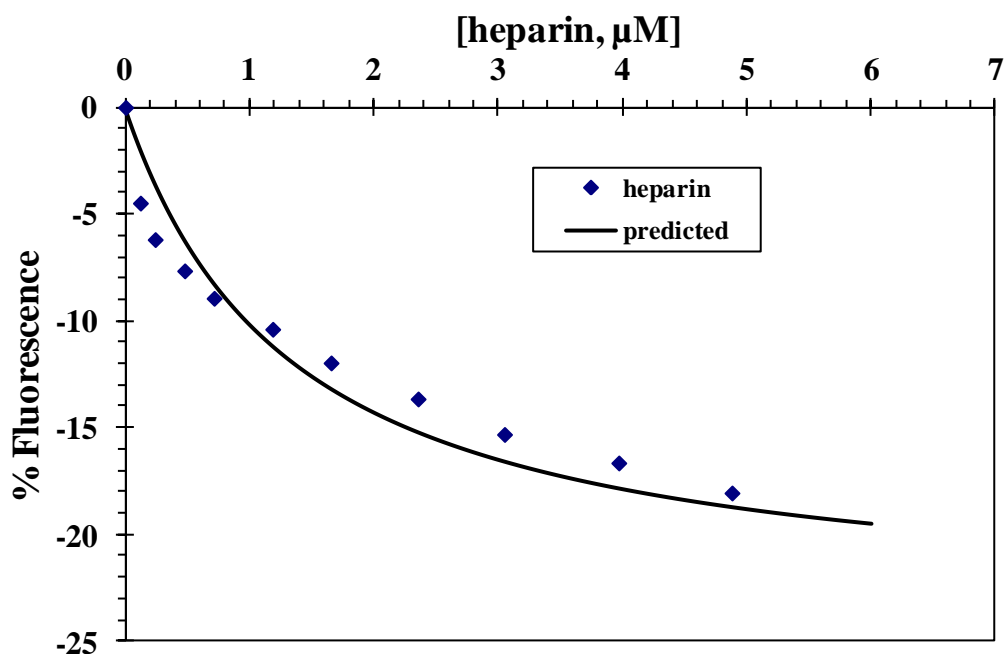
Figure 36: K_D of heparin for FXIa based on changes in intrinsic fluorescence

4.2.4 Results

The IC_{50} profile of heparin against FXIa indicates that heparin is indeed binding and inhibiting FXIa and its IC_{50} was found to be 0.21 ± 0.05 μM. Heparin inhibited FXIa by an inhibition of 30%.

4.3 Effect on inhibitory profiles of compounds 13 and 24 in presence of heparin

In order to ascertain whether our compounds were competing with heparin, the inhibition profiles for both compounds (**13** and **24**) were determined by incubating FXIa with heparin as well as the compounds.

4.3.1 Materials:

The materials and chemicals used were the same as those mentioned in section 3.2 (Materials section)

4.3.2 Observations and inhibitory profiles

Serial dilutions of both the compounds **13** and **24** were made such that every dilution was $5/6^{th}$ the concentration of the previous. Three solutions of different concentrations (414nM, 1.2 µM, 2.4 µM) of heparin-Na^+ (average molecular weight ~ 15000) were prepared in water. The final concentration of FXIa was 1.53nM while that of substrate was 345 µM. The assay consisted of adding 75 µL of Buffer C followed by 5 µL of inhibitor (compounds **13** or **24**) and 10 µL of FXIa and 10mins incubation at 37°C. After incubation, 5 µL of heparin-Na^+ was added followed by an incubation of 5mins after which 5 µL of substrate was added. The initial rate of substrate hydrolysis by FXIa was measured at 405nm using FlexStation III (Molecular Devices, Sunnyvale, CA). Three inhibition profiles of the inhibitors at different heparin-Na^+ concentrations and one in the absence of heparin-Na^+ were generated.

The inhibition profiles for **13** and **24** are given in **figures 37** and **38**.
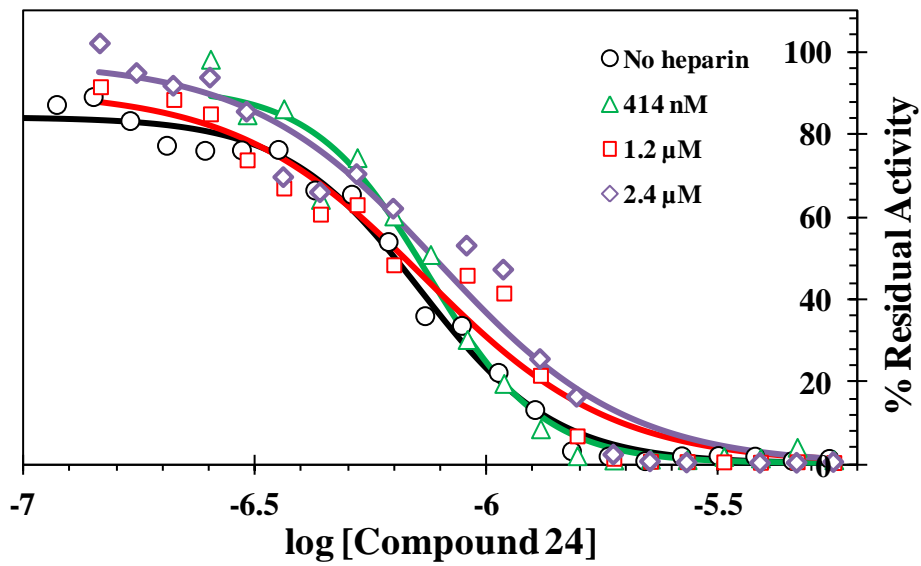
Figure 37: Inhibition profiles of compound 24 in absence and presence of three different concentrations of heparin-Na$^+$
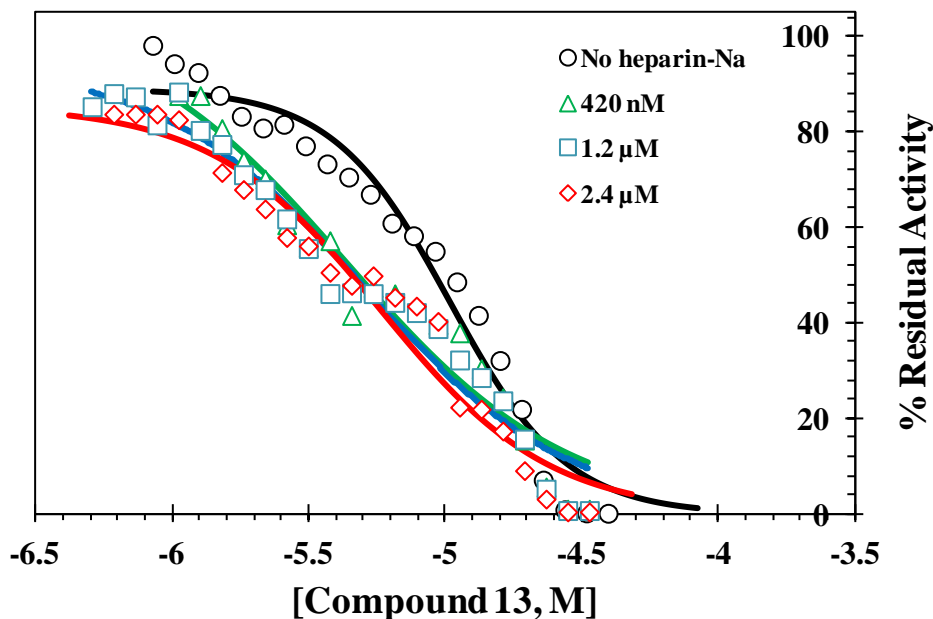
Figure 38: Inhibition profiles of compound 13 in absence and presence of three different concentrations of heparin-Na$^+$

4.3.3 Results:

[heparin-Na$^+$]	Compound 24 IC$_{50}$ μM	Compound 13 IC$_{50}$ μM
0	0.7 ± 0.03	10.3 ± 0.7
420 nM	0.74 ± 0.04	4.3 ± 1.7
1.2 μM	0.73 ± 0.11	5.1 ± 0.8
2.4 μM	0.8 ± 0.08	5.83 ± 0.6

Table 10: IC$_{50}$ of compounds 24 and 13 in presence of varying concentrations of heparin

From **figures 37** and **table 10** it can be seen that with increasing concentrations of heparin-Na$^+$ the IC$_{50}$ profiles for compound 24 did not change significantly. This indicates that perhaps compound **24** is not competing with heparin-Na$^+$ and may have a unique binding site of

its own which is different than the known binding site of heparin. The IC_{50} profiles of compound **13 (Figure 38)** shows a noticeable decrease in IC_{50} from ~ 10 µM to ~5 µM. This could be an indication that although this compound does not compete with heparin for FXIa, it could be acting in a synergistic manner. However, further analysis needs to be done in order to confirm this.

4.4 Conclusions and Future directions:

FXIa is an unusual serine protease in the coagulation cascade not only in terms of its structure but also its behavior. Compared to the other more popular targets, FXIa has not been studied as extensively. Hence it is difficult to extrapolate the results and behaviors of other known targets to FXIa. Of late it has been gaining recognition as a possible new target for anti-thrombotic drugs and is reported to be a safer target. The aim of this project was to find an inhibitor from a small-sulfated molecule library and to study its mode of inhibition and interaction with FXIa. Out of the library screened, compounds **13** and **24** seemed to show promising results as FXIa inhibitors. These compounds were found to bind at a site other than the active site based on the results obtained from Michaelis-Menten kinetics as well as fluorescence quenching. Although these compounds seem to be allosteric inhibitors, there are other questions which remain to be answered, one of them being the stoichiometry with which these compounds bind with FXIa. This data will help in understanding how the molecules interact with FXIa. Since the enzyme has two subunits it will be interesting to observe how these inhibitors interact with FXIa and to know whether FXIa exhibits cooperativity. The IC_{50} profiles of both **13** and **24** have shown biphasic curves and this phenomenon has to be investigated further.

69

Molecular docking studies: Heparin is proposed to bind to three different locations on FXIa of which one is on the apple-3 domain and the other two are on the catalytic domain. Results from this project indicate that **13** and **24** do not compete with heparin for FXIa. This may mean that these compounds bind at a site that is different than that of the heparin binding site. How well these molecules can be docked to the heparin-binding site can provide more information that could supplement the results obtained from the assays.

In conclusion, amongst all the compounds which were screened against FXIa from the mini-library two compounds were chosen, a dimer and a trimer of the same benzofuran scaffold.

X-ray crystallography: In the future, crystal structures of one of the inhibitors with FXIa will help in understanding the mode of interaction of these molecules as well as the residues with which they interact.

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