CHAPTER 3

Molecular docking study and biological activities of 2-substituted benzamidobenzene derivatives as factor Xa inhibitors

3.1 Abstract

Chapter 3 deals with the biological activities of the novel 2-substituted benzamidobenzene derivatives synthesized in chapter 2 which includes human factor Xa inhibitory activity. Thrombin inhibitory activity and anticoagulant activity using prothrombin and activated partial thromboplastin time (PT and APTT) assay were also determined for those compounds which showed good fXa inhibitory activity. The docking result of the most active compound is also discussed herein.

3.2 Introduction

Molecular docking is one of the most commonly used computational tool commonly applied in drug discovery projects and fundamental biological studies for molecular interactions, mainly receptor-ligand interactions. With more complex molecular mechanics program, it is possible to superimpose the three dimensional structure of a potential drug like candidate on its possible target site. This process, which is often automated, is known as docking.

The method is widely used in the field of structure-based drug design, where researchers try to find compounds, which will form a low energy stable intermolecular complex with a target protein. Initial screening of possibly millions of compounds in a laboratory is often too expensive and time-consuming process to be feasible and thus molecular docking methods are used to quickly eliminate unlikely candidates without executing their synthesis.[1-3]

The docking process involves the prediction of ligand conformation and orientation within a targeted binding site. In general, there are two aims of docking studies: accurate structural modeling and correct prediction of activity. The binding modes of two interacting molecules based on their topographic features or energy-based considerations are explored using molecular docking and it aims to fit them into conformations that lead to favourable interactions.[4] Thus docking is one of the most important tool in determining the active conformation of a drug, i.e. its conformation when bound to the receptor. Hence, prediction of binding orientations of small molecules in a protein binding site has become increasingly important in drug design. Identification of leads is driven either by random screening or a directed design approach, and traditionally both strategies have been of equal importance, depending on the problem in hand. The directed design approach needs a rational starting point for medicinal chemists and molecular modeling scientist to exploit. Examples include the design of analogs of a drug known to be active against a target receptor and mimics of the natural substrate of an enzyme. Increasingly, the three-dimensional structure of many biological targets is being revealed by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, opening the way to the design of novel molecules that directly exploit the structural characteristics of the receptor binding site. This approach of structure-based design has a major impact on the

rational design and optimization of new lead compounds in those cases where the receptor structure is well characterized.[5-7]

Molecular docking can be thought of as a lock-and-key process where a protein can be considered as a lock and the ligand as a key. Here one is interested in finding the correct relative orientation of the key which will open up the lock. This is the simplest to simulate, but is generally thought to be unreasonable. However since both the ligand and protein are flexible, a hand-in-glove analogy is more appropriate than a lock-and-key.[8] During the course of process, the ligand and the protein adjust their conformation to achieve an overall best fit and this kind of conformational adjustments resulting in the overall binding is referred to as a induced-fit.[9]

Theory of Docking

Molecular docking gives a prophecy of the ligand-receptor complex structure using computation methods. Docking can be achieved by two steps which are interrelated to each other.

1) Sampling conformations of the ligand in the active site of the protein and

2) Ranking these conformations via a scoring function.

Ideally, sampling algorithms should be able to reproduce the experimental binding mode and the scoring function should also rank it highest among all generated conformations.

To evaluate various docking methods, it is important to consider how the protein and ligand are represented. There are three basic representations of the receptor: atomic, surface and grid.[10] Among these, atomic representation is generally only used in conjunction with a potential energy function[11] and often only during final ranking procedures.

Surface-based docking programs are used in protein–protein docking but not frequently.[12,13] Great extent of research in this area was initiated by Connolly's early work on molecular surface representations.[14,15] These methods attempt to align points on surfaces by minimizing the angle between the surfaces of opposing molecules[16]. Therefore, a rigid body approximation is still the standard for many protein–protein docking techniques.

Goodford was the first to establish the use of potential energy grids[17] and various docking programs use such grid representations for energy calculations. The basic idea is to store information about the receptor's energetic contributions on grid points so that it only needs to be read during ligand scoring. In the most basic form, grid points store two types of potentials: electrostatic and van der Waals.

1) Sampling Algorithms

Most docking algorithms consider only the fexibility of the ligand when searching through the space of admissible conformations. While some docking packages allow to account for receptor flexibility, either side-chains or backbone, in many cases the benefit of modeling flexibility of both receptor and ligand is marginal due to the exponential increase in searching space. However, some docking algorithms allow dealing with limited local flexibility either by rotating or flipping some moieties or using soft" energetic terms.[18,19] Treatment of ligand flexibility can be divided into three basic categories[20] (i) exhaustive search, (ii) stochastic search, and (iii) simulation.

(i) Exhaustive

Performing an exhaustive search through the whole conformational space quickly becomes infeasible due to the exponential growth with respect to the number of rotational bonds. Thus, most algorithms use a branch-and-bound approach to reduce the conformational space. The geometric and chemical properties of the binding site are limiting factors that allow to reject many conformations. Some algorithms incrementally build the ligand structure within the cavity of the receptor, by first posing a rigid core of the ligand and then linking its other parts while complying with the geometric constraints of the binding site.[21] Alternatively, the ligand may be divided into rigid fragments, each of which is docked separately into the receptor. Then, rigid fragments are tethered using the flexible parts of the ligand.[18]

(ii) Stochastic

The two dominant approaches in this category are tabu search and genetic algorithms. In tabu search, small random perturbations are applied to the current conformation followed by their ranking according to some fitness function that in many cases only evaluates geometric constraints (to increase performance). Rejected conformations are marked (tabu) to avoid re-exploring them. In the genetic approach,

a population of conformations is evaluated at each iteration and a set of random perturbations (mutations") and breeding rules are applied to the most fitted conformations in order to improve the fitness of the next generation.[22,23]

(iii) Simulation

Simulation methods are generally based on molecular dynamics and are being used in two ways. One to sample a diverse set of low-energy (local minima) conformations, where diversity, in this case, is usually measured by RMSD distance from all current selected conformations. Or two, in complement to another searching method, as a post-processing stage.[24]

2) Scoring Functions

The strength of non-covalent interaction between two molecules after they have been docked are predicted by a mathematical methods known as scoring functions. Most commonly one of the molecules is a small organic compound designed as a drug and the other is drugs biological target such as a protein.[25] The scoring function takes a pose as input and returns a number indicating the probability that the pose represents a favorable binding interaction. Most scoring functions are physics based molecular mechanics force fields that estimate the energy of pose as a low energy (negative) indicates a stable system and thus a likely binding interaction. Scoring functions implemented in docking algorithms tend to simplify or ignore complex physical and chemical terms such as entropy or hydrophobicity. Scoring functions can be divided into three categories as shown in **Figure 3.1.**

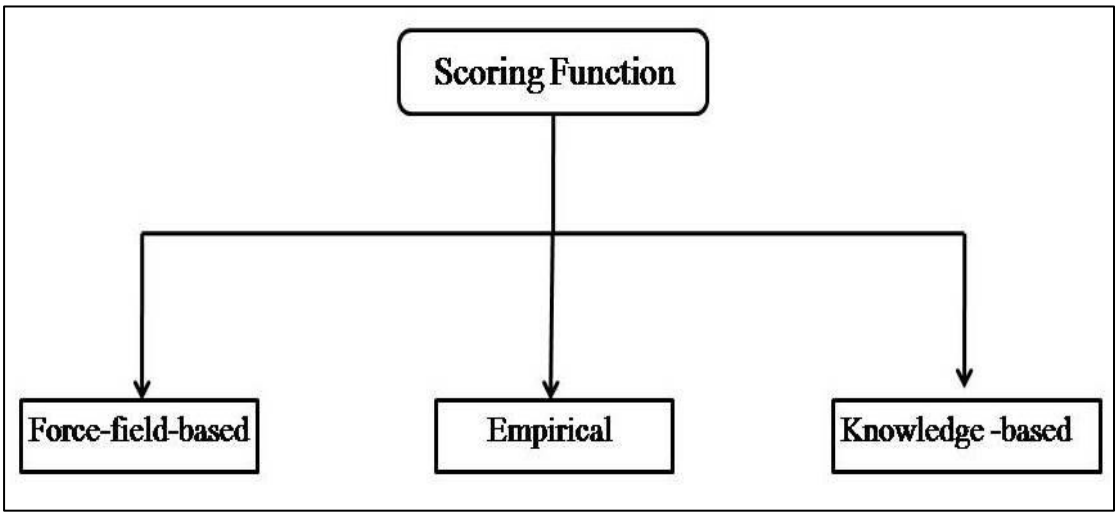


Figure 3.1 Types of Scoring Functions

Force-field-based scoring functions evaluate the binding energy by accounting for the non-bonded (electrostatics and van der Waals) interactions between all pairs of atoms. The electrostatic terms are calculated by a Coulombic formulation.[26-28]

Empirical function is based on counting the number of various types of interactions between a ligand and a receptor.[29] Binding energy decomposes several energy components, such as hydrogen bond, ionic interaction, hydrophobic effect and binding entropy. Each component is multiplied by a coefficient and then summed up to give a final score. Coefficients are obtained from regression analysis fitted to a test set of ligand-protein complexes with known binding affinities.

Knowledge-based methods use statistical analysis of ligand-protein complexes crystal structures to obtain the inter-atomic contact frequencies and/or distances between the ligand and protein. These methods employ the assumption that highly observed types of interactions are energetically favorable.[30]

Virtual screening is a computational technique used in drug discovery research. It involves the rapid *in silico* assessment of large libraries of chemical structures in order to identify those structures which are most likely to bind to a drug target, typically a protein receptor or an enzyme.[31] It uses large number of docking program and each program has different algorithms to handle ligand and protein flexibility, scoring functions and CPU time to dock a molecule to a given target. When the ligand and the target are treated as rigid bodies in the docking process,[32] then the conformational flexibility of ligands can be taken into account by creating a collection of conformers and docking each one of them separately into target site or it can also be explored during the docking process. Incremental growth methods,[33] genetic algorithm (e.g. GOLD),[34] Tabu search (e.g. PRO_LEADS)[35] and combined Monte Carlo and simulated annealing methods (e.g. Dock Vision)[36] are the examples of semi-flexible docking approaches. Practically binding of a ligand to a receptor site can induce large conformational changes thus performing docking studies using rigid protein is sometimes an inaccurate approximation.[37,38] To overcome this problem use of an ensemble of protein conformers can be made. Example of such a docking program is FlexE, wherein various protein conformers are superimposed and it treats the dissimilar protein regions as distinct alternatives.[39] Once a pose has been

generated for a ligand in the binding site, scoring function needs to be applied to rank the quality of the pose with respect to other poses of the compound based on binding energy of association of each pose. Scoring function estimates the free energy of binding of a ligand in a target-ligand complex. There are wide choices of scoring functions available as discussed above (force-field based, empirical and knowledge-based functions). It is a well known fact that these fast scoring methods do not perform so accurately as the time-consuming free energy perturbation technique.[40] Using a combination of one or more scoring functions (i.e. consensus scoring) has been reported to improve the results.[41] AUTODOCK,[42] CDOCKER,[43] DOCK,[44] FlexX,[45,46] GOLD,[47] GLIDE[48,49] are some examples of docking programs used in drug design.

Factor Xa Enzymatic Activity

Enzymes are the proteins that catalyze most of the chemical reactions that take place in the body. They make it possible for chemical reactions to occur at neutral pH and body temperature. The chemical compound upon which the enzyme exerts its catalytic activity is called a substrate. Proteolytic enzymes act on their natural substrates, proteins and peptides by hydrolyzing one or more peptide bond(s). This process is usually highly specific in the sense that only peptide bonds adjacent to certain amino acids are cleaved.

Factor X is a key substance in the series of reactions leading to the coagulation of blood. The activation of factor X brings about the formation of the proteolytic enzyme, factor Xa, which is directly responsible for the transference of prothrombin to thrombin. The transformation of prothrombin to thrombin by factor Xa involves the cleavage of two peptide bonds in the prothrombin molecule. The detailed mechanism is already discussed in chapter 2 (**Scheme 2.1**). These two cleavage sites are preceded by exactly the same amino acid sequence: -Ile-Glu-Gly-Arg- (**Figure 3.2**). *Invitro* determination of factor Xa is done using chromogenic substrate with an amino acid sequence, -Ile-Glu-Gly-Arg-, corresponding to the sequence preceding the cleavage sites of the natural substrate prothrombin. Chromogenic substrates are peptides linked to chromopore that react with proteolytic enzymes under the formation of color. They are made synthetically and are designed to possess selectivity similar to that of the natural substrate for the enzyme. Attached to the peptide part of the chromogenic

substrate is a chemical group which when released after the enzyme cleavage gives rise to color. The color change can be followed spectrophotometrically and is proportional to the proteolytic activity.[50]

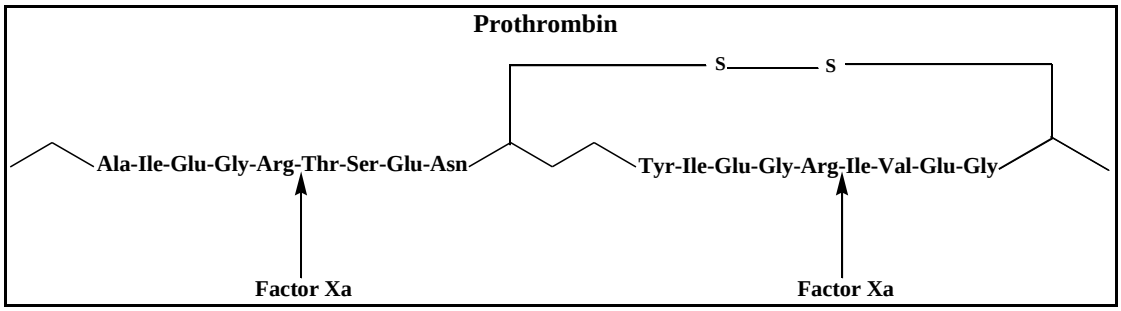


Figure 3.2 Cleavage of Prothrombin by proteolytic enzyme factor Xa

Chromogenic substrate technology was developed in the early 1970s, and has since then become a tool of substantial importance in basic research. The substrate Benzoyl-lle-Glu-Gly-Arg-p-nitroanilide (S-2222) (**Figure 3.3**) has an amino acid sequence identical to the natural substrate of prothrombin.[51]

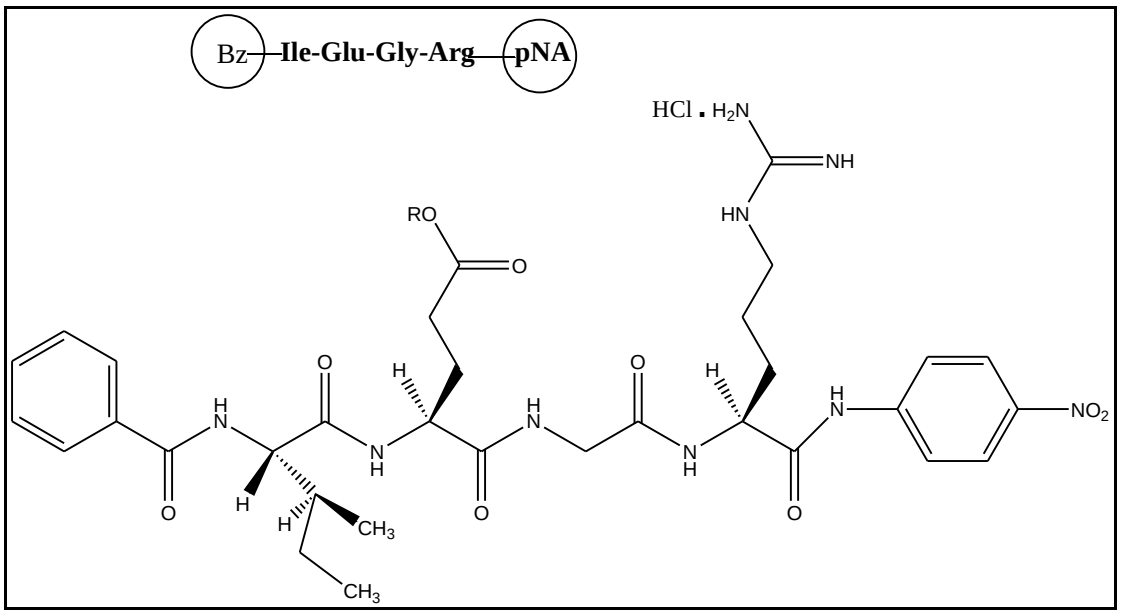
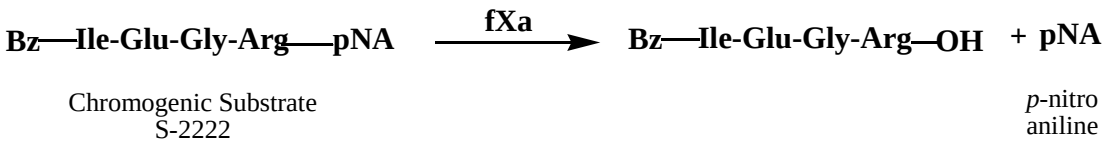


Figure 3.3 Structure of Chromogenic substrate (S-2222)

This sequence of residue mimics the sequence of the natural substrate. The hydrolysis of substrate causes the release of *p*-nitro aniline as shown in **scheme 3.1.**



Scheme 3.1 Cleavage of chromogenic substrate using factor Xa

The released *p*-nitroaniline (pNA) has a light absorption maximum different from that of the substrate, and the enzymatic reaction can easily be followed by measuring the increase in absorption at 405 nm, which is proportional to the amount of active factor Xa.[51]

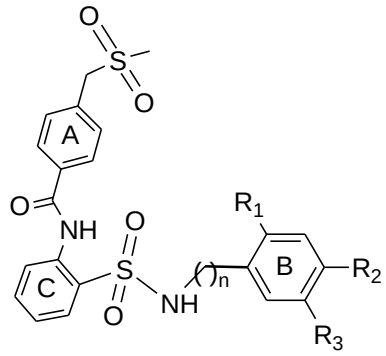
In this chapter the human factor Xa inhibitory activity of the novel 2-substituted benzamidobenzene derivatives synthesized in chapter 2 has been discussed. Thrombin inhibitory activity and anticoagulant activity using prothrombin and activated partial thromboplastin time (PT and APTT) assay are also reported for those compounds which showed good fXa inhibitory activity. The docking result of the most active compound is also discussed herein.

3.3 Results and Discussion

The compounds synthesized in chapter 2 were initially screened using two concentrations of the compounds (500 µM and 100 µM) after which IC_{50} values were determined for only those compounds that led to approximately 50 % reproducible inhibition of the coagulation enzyme at 100 µM.

The human factor Xa inhibition activity of **30a-30g** (**Scheme 2.2**, **Chapter 2**) having sulfone group at S4 ligand and sulfonamide linker connecting S1 ligands were studied (**Table 3.1)**. It was found that, **30a** having no substituents in ring B displays 37 % inhibition of fXa activity at 500 µM while insertion of methylene group between phenyl ring and sulfonamide group in **30b**, led to decrease in fXa inhibition activity (9 % inhibition). Methyl and bromo substituents in 4th position of ring B in **30c** and **30d** showed 23 % and 38 % inhibition of fXa activity. Introduction of a methoxy group at 2 and 5 positions and a chloro group at 4th position in **30e** showed no inhibition. Replacement of ring B with 4-fluorophenyl and 4-methoxyphenyl afforded inactive compounds (**30f** and **30g**).

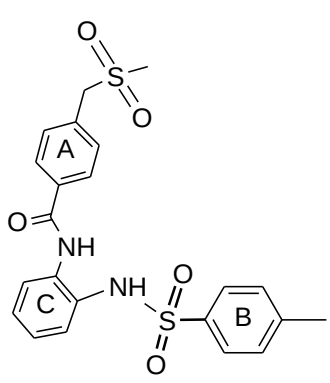
Table 3.1 Effect of B ring substituents on fXa inhibition



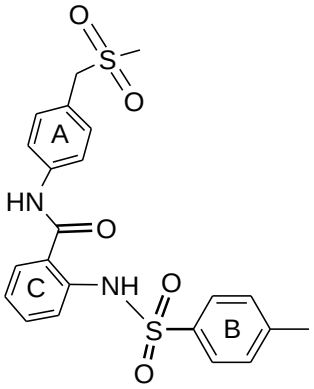
Compound	R_1	R_2	R_3	n	fXa (% inhibition at 500 µM)
30a	H	H	H	0	37
30b	H	H	H	1	9
30c	H	CH_3	H	0	23
30d	H	Br	H	0	38
30e	OCH_3	Cl	OCH_3	0	No inhibition
30f	H	F	H	0	No inhibition
30g	H	OCH_3	H	0	No inhibition

We have also investigated the effect of interchange of ring A and ring B linkers (**Scheme 2.3**, **Chapter 2**) on fXa inhibitor activity as shown in **Table 3.2**. Inversion of ring B linker as in **40** resulted in diminished potency (18% inhibition) while **41** with both amide and sulfonamide linker connected to rings A and B reversed, showed no fXa inhibitory activity.

Table 3.2 Effect of inverted amide and sulfonamide linkers on the activity



40

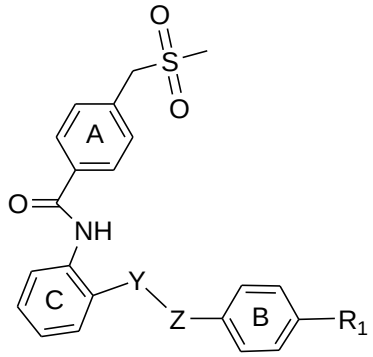


41

Compound	fXa (% inhibition at 500 µM)
40	18
41	No inhibition

In order to determine the structural requirement for fXa activity, alternative linkers to sulfonamide were explored (**Scheme 2.4**, **Chapter 2**). As the data in **Table 3.3** indicates, compound (**48a**) containing an ether linker displayed 84 % inhibition of fXa at 500 µM but showed no inhibition at 100 µM concentration, while compounds with thioether linkers (**48b** and **48c**) resulted into inactive compounds. **48d** having methylsulfonylmethyl linker showed no inhibition while the introduction of methylsulfonyl linker as in **48e** led to 41 % fXa inhibition of the enzyme activity at 500 µM.

Table 3.3 Alternative sulfonamide linkers and their effect on activity

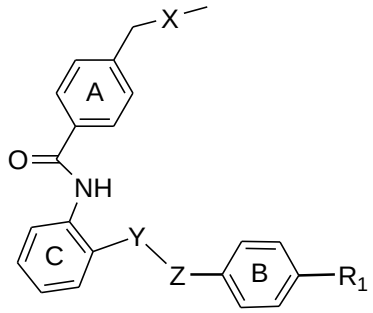


Compound	Y	Z	R_1	fXa (% inhibition at 500 µM)
48a	CH_2	O	H	84[a]
48b	CH_2S	CH_2	H	No inhibition
48c	CH_2	S	H	No inhibition
48d	CH_2SO_2	CH_2	H	No inhibition
48e	CH_2	SO_2	H	41

[a]No inhibition at 100 µM

We then decided to replace sulfone group at S4 ligand (4^{th} position of ring A) by sulfoxide and sulfide groups in some of the designed molecules to observe their effect on fXa activity (**Table 3.4**). Replacement of sulfone group in **30d** by sulfoxide and sulfide resulted in **49c** and **50c** with improved fXa inhibition activity of 72 % and 100 % inhibition at 500 µM concentration while offering 66 % and 45 % inhibition at 100 µM. Similarly, **49a** having methylsulfonylmethyl as S1 ligand (ring B) linker with the sulfoxide group at S4 ligand showed somewhat higher inhibition as compared to **48d** having sulfone group while **50a** with sulfide group exhibited sudden augmentation in activity to 100 % inhibition at 500 µM and 26 % at 100 µM. Compounds **49b** and **50b** having methylsulfonyl linker with sulfoxide and sulfide groups at S4 ligand (4^{th} position of ring A) showed comparable inhibition of fXa with that of **48e** indicating that the presence of sulfonamide groups as S1 ligand (ring B) linkers and sulfide or sulfoxide groups at S4 ligand (4^{th} position in ring A) is favorable for this activity as compared to sulfone.

Table 3.4 Sulfides and sulfoxides as S4 binding elements and their effect on biological activity



Compound	X	Y	Z	R_1	fXa (% inhibition at 500 µM)	fXa (% inhibition at 100 µM)	Thrombin (% inhibition at 100 µM)
49a	SO	CH_2SO_2	CH_2	H	28	nd[a]	nd[a]
49b	SO	CH_2	SO_2	H	47	nd[a]	nd[a]
49c	SO	SO_2	NH	Br	72	66	21
50a	S	CH_2SO_2	CH_2	H	100	26	3
50b	S	CH_2	SO_2	H	50	25	nd[a]
50c	S	SO_2	NH	Br	100	45	No inhibition

[a] Not determined

IC_{50} values for **49c** and **50c** were determined (**Table 3.5**) as discussed in experimental section. Chromogenic substrate hydrolysis assay was used to measure direct inhibition of fXa as previously reported.[52] This assay results in a linear augmentation in absorbance at 405 nm due to substrate hydrolysis caused by fXa. The residual enzyme activity is the attribute of the slope. The change in fXa residual activity as a function of the concentration of the potential inhibitors is plotted on a logarithmic scale (**Figure 3.4**) and fitted by the logistic dose-response relationship (**Eq. 3.1**) to determine the potency (IC_{50}), efficacy ($\Delta Y = Y_M - Y_0$) and Hill Slope (*HS*) of inhibition.[53]

Table 3.5 fXa inhibition features for compounds (**49c and 50c**)

Compound	IC_{50} (µM)	*HS*	ΔY
49c	29.2 ± 2.3	1.3 ± 0.2	70.9 ± 4.6
50c	16.1 ± 1.4	1.1 ± 0.2	75.3 ± 4.9

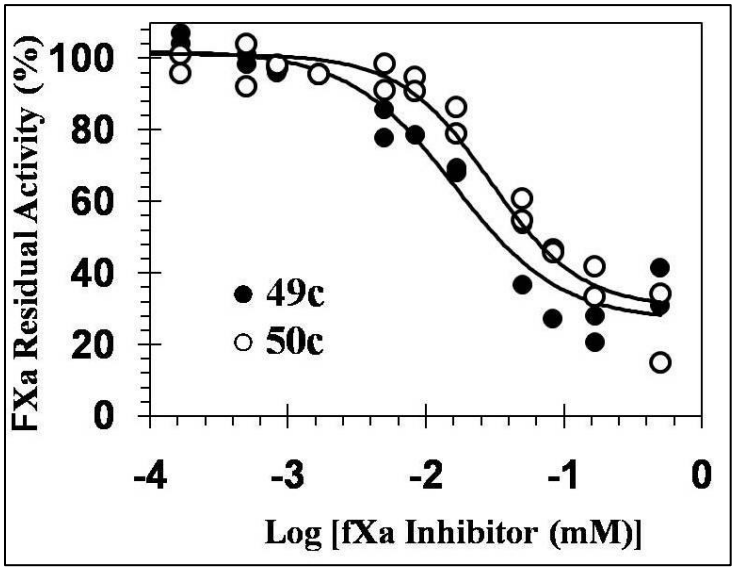


Figure 3.4 Direct inhibition of fXa by **49c** and **50c**. Solid lines represent sigmoidal dose-response fits to the data to obtain the values of IC_{50}, Y_M, Y_0, and *HS*, as described in the experimental section

Selectivity against thrombin was also examined for a few representative candidates **49c**, **50a**, and **50c** which showed good fXa inhibitory activity. All these members evaluated for this activity showed either very poor or no inhibition at 100 µM concentration (**Table 3.4**). Compounds (**49c** and **50c)** selected on the basis of fXa inhibition data were also tested for anticoagulant activity using prothrombin and activated partial thromboplastin time (PT and APTT) assay. These compounds failed to double the PT and APTT time, even at 2000 µM concentration (**Table 3.6**). This may be attributed to their poor solubility and high lipophilicity resulting in high plasma protein binding. Previously, similar results have also been reported by many research groups where the PT and APTT assays did not correlate with activity against fXa or thrombin.[54-56]

Table 3.6 Human Plasma clotting assays

Compounds[a]	Clotting time in APTT (sec)	Clotting time in PT (sec)
DMSO	35.9 ± 1.3	20.4 ± 0.9
49c	48.7 ± 0.8	37.9 ± 1.9
50c	41.9 ± 10.7	24.4 ±1.2

[a]2000 µM concentration

Docking studies

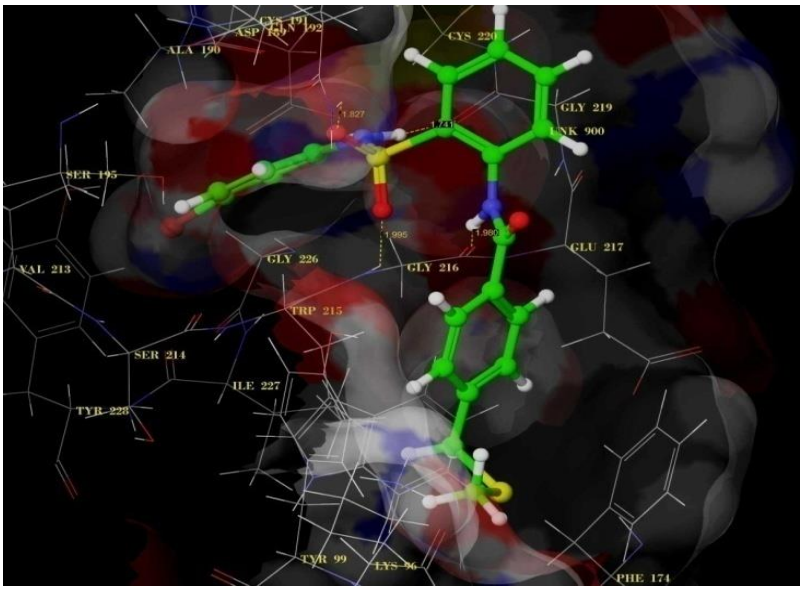


Figure 3.5 Compound (**50c**) docked into the active site of fXa

Factor Xa (fXa) has a well-recognized active site comprising of mainly four regions. These are S1, S2, S4, and an ester binding pocket (EBP) in the active binding site. The S1 and S4 sites are more important for ligand binding while the S2 is a small sack separated from S4 by Tyr 99 (Nazare et al. 2012). Docking studies of the synthesized compounds were performed using Glide[57] with extra precision (XP) mode. Before docking of the actual synthesized compounds, the generated grid on fXa receptor (PDB Code: 4A7I)[58] was validated by re-docking the co-crystallized ligand. Very similar interactions between ligand and receptor were observed. The RMSD value of 0.36 Å was observed between re-docked ligand and the original coordinates of the ligand.

Here the docking interactions between the most active compound (**50c**) with the active site of fXa is explained and is reproduced in **Figure 3.5**. The bromophenyl group shows good lipophilic interactions with the S1 sub-pocket of active site. The non-covalent lipophilic interaction between Br and π electron system of Tyr228 of S1 site appears to further stabilize the ligand-receptor complex which is located at ~ 3.7 Å from the centroid of Tyr228 aromatic ring. NH of SO_2NH group imparts stability to the ligand-receptor complex by forming hydrogen bonded interaction with C=O of Gly219 (1.74 Å). The SO_2 group of the ligand also interacts with the receptor by

means of two hydrogen bonds, one with NH of Gln192 (1.83 Å) and the other with NH of Gly216 (1.99 Å). The amide NH of the ligand on the other hand provides stability by forming hydrogen bonding with C=O of Gly216 (1.98 Å) of the receptor. The benzyl methyl sulfide group of the ligand fits into the S4 sub-pocket of the active site with centroid to centroid distance of ~4.7 Å, ~5 Å and ~5.9 Å between the aromatic part of the ligand and Tyr99, Phe174 and Trp215 respectively resulting into good van der Waals interaction among these moieties.

3.4 Experimental

Biology

Human antithrombin (AT) and human coagulation factors Xa and IIa were purchased from Haematologic Technologies (Essex Junction, VT). Stock solutions of proteins were prepared in 20 mM Tris-HCl buffer, *p*H 7.4, containing 100 mM NaCl and 2.5 mM $CaCl_2$, 0.1 % polyethylene glycol (PEG) 8000 and 0.02 % Tween 80. Chromogenic substrates Spectrozyme TH was purchased from American Diagnostica (Greenwich, CT), while substrate S-2222 was from DiaPharma Group, Inc (West Chester, OH).

fXa and Thrombin inhibition studies

Measurement of direct fXa and thrombin inhibition was done using a chromogenic substrate hydrolysis assay as reported earlier[53] using a microplate reader (FlexStation III, Molecular Devices). Initial screening was done using two concentrations of the compounds (500 uM and 100 uM) after which IC_{50} values were determined for compounds that led to >50 % reproducible inhibition of coagulation enzyme in the initial screening. Relative residual enzyme activity at each concentration of the inhibitor was calculated from the ratio of the enzyme activity in the presence and absence of the inhibitor. For IC_{50} determination, stocks of potential inhibitors were serially diluted to give 18 different aliquots in the wells with final concentrations ranging from 500 – 0.000833μM.

For fX*a* inhibition studies incubation was done at 37 °C and in *p*H 7.4 buffer containing 20 mM Tris-HCl, 2.5 mM $CaCl_2$, 100 mM NaCl, 0.1 % polyethylene glycol (PEG) 8000 and 0.02 % Tween 80 was used. 180μL of pH 7.4 buffer was

added to the wells, and 5µL of potential fXa inhibitor (or solvent reference) and 10µL of fXa (10nM final concentration) were consecutively added. Incubation was done for 10 minutes after which, 5 µL of fXa substrate (125µM) was added rapidly and the fXa residual activity was measured from the initial rate of increase in absorbance at 405 nm. The ratio of fXa activity in the presence and absence of inhibitor was used to calculate the relative residual fXa activity at each concentration of the inhibitor. To obtain the potency (IC_{50}) and efficacy (ΔY) of inhibition, logistic **Eq. 3.1** was used to fit the dose dependence of residual protease activity.

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log [I]_0 - \log IC_{50})(HS)}} \qquad (3.1)$$

In this equation, Y is fractional residual factor Xa activity in the presence of inhibitor to that in its absence, Y_M is the maximum possible value of the fractional residual factor Xa activity and Y_0 is the minimum possible value of the fractional residual factor Xa activity respectively, IC_{50} is the inhibitors concentration that results in 50% inhibition of enzyme activity, and HS is the Hill slope. Nonlinear curve fitting resulted in Y_M, Y_0, IC_{50}, and HS values.

Thrombin inhibition studies was conducted at 25 °C and the buffer used was 20 mM Tris-HCl buffer, *p*H 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1 % polyethylene glycol (PEG) 8000. Generally, 192µL of *p*H 7.4 buffer was added to the wells, and 1µL of potential thrombin inhibitor (or DMSO) and 5µL of thrombin (6nM final concentration) were sequentially added. After a 10 min incubation, 5 µL of thrombin substrate (125µM) was added rapidly and the residual thrombin activity was measured from the initial rate of increase in absorbance at 405 nm. Relative residual thrombin activity was calculated as with f*Xa* above.

Prothrombin time (PT) and activated partial thromboplastin time (APTT) in human plasma.

Clotting time was measured using a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparles, MD) in a standard one-stage re-calcification assay as described previously.[59] For PT assays, thromboplastin was reconstituted according to manufacturer's directions and warmed to 37 °C. 10 µL sample of the compound was

added to 90 μL citrated human plasma to give the desired concentration which was then incubated for 30 s at 37 °C. 200 μL pre-warmed thromboplastin was added to it and the time to clot was recorded. In the absence of an inhibitor clotting time was determined using 10 μL of DMSO. For the APTT assay, 10 μL of sample was mixed with citrated human plasma (90 μL) and pre-warmed APTT reagent (100 μL) (0.2% ellagic acid). After 4 min of incubation, clotting was initiated by addition of 25 mM $CaCl_2$ (100 μL) (37 °C) and time to clot was noted. Each clotting assay was performed in duplicate or triplicate.

Docking Studies

Docking studies were performed using Glide tool of Schrödinger 2009.[57] It executes grid-based ligand docking and looks for favorable interactions between the ligand and the receptor. The 3D structures of ligands were built within Maestro using the Build module and a single low energy conformation search was carried out for all molecules using OPLS_2005 force field at physiological *p*H condition using LigPrep module of Schrödinger2009. The 3D crystallographic structure for factorXa (fXa) was obtained from RCSB Protein Data Bank (PDB Code: **4A7I**). Docking calculations for energy optimized 3D ligands were performed in extra precision (XP) mode with the active sites of receptor structure.

3.5 Conclusion

In conclusion compounds (**49c** and **50c**) showed good fXa inhibitory activity amongst the synthesized derivatives in the series. Presence of sulfonamide linker was found to be optimal of all the linkers studied. A significant increase in potency was observed by the introduction of sulfide or sulfoxide group in place of sulfone group. IC_{50} value for **49c** was found to be 29.2 μM with an efficacy of 70.9 %, while that for **50c** was 16.1 μM with efficacy of 75.3 %. The *HS* for inhibition was found to be 1.3 for **49c** and 1.1 for **50c**. Compounds (**49c** and **50c)** showing good fXa inhibition were found to show good selectivity for fXa over thrombin suggesting they have the potential to discriminate between fXa and other closely related serine proteases. To establish a probable mechanism of action of the synthesized compounds as fXa inhibitors, docking studies have also been performed.

3.6 References

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