Pharmacophore Identification, in Silico Screening, and Virtual Library Design for Inhibitors of the Human Factor Xa

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Factor Xa inhibitors are innovative anticoagulant agents that provide a better safety/efficacy profile compared to other anticoagulative drugs. A chemical feature-based modeling approach was applied to identify crucial pharmacophore patterns from 3D crystal structures of inhibitors bound to human factor Xa (Pdb entries 1fjs, 1kns, 1eqz) using the software LIGANDSCOUT and CATALYST. The complex structures were selected regarding the criteria of high inhibitory potency (i.e. all ligands show K_i values against factor Xa in the subnanomolar range) and good resolution (i.e. at least 2.2 Å) in order to generate selective and high quality pharmacophore models. The resulting chemical-feature based hypotheses were used for virtual screening of commercial molecular databases such as the WDI database. Furthermore, a ligand-based molecular modeling approach was performed to obtain common-feature hypotheses that represent the relevant chemical interactions between 10 bioactive factor Xa inhibitors and the protein, respectively. In a next step a virtual combinatorial library was designed in order to generate new compounds with similar chemical and spatial properties as known inhibitors. The software tool ILIB DIVERSE was used for this procedure in order to provide new scaffolds of this group of anticoagulants. Finally we present the combination of these two techniques, hence virtual screening was performed with selective pharmacophore models in a focused virtual combinatorial database. De novo derived molecular scaffolds that were able to adequately satisfy the pharmacophore criteria are revealed and are promising templates for candidates for further development.

INTRODUCTION

The prevention of blood coagulation is a major topic considering serious physiological reactions of undesired blood clotting that can lead to myocardial infarction, stroke, deep vein thrombosis, and pulmonary embolism. Factor Xa is a trypsin-like serine protease that converts the prothrombin zymogen to its active form thrombin and initiates the final stage of the blood coagulation pathway. Thrombin catalyzes enzymatic reactions that result in the formation of fibrin, the important binding part for blood clotting. Compared to thrombin, factor Xa acts on an earlier level than thrombin. The mechanism of amplification in the blood coagulation cascade causes a small amount of factor Xa to produce a large amount of thrombin. Therefore, the direct inhibition of factor Xa is considered as more favorable regarding the safety/efficacy ratio compared to other anticoagulants. Other anticoagulant substance classes such as vitamin K antagonists or thrombin inhibitors are considered to have a higher bleeding risk than factor Xa inhibitors. Hence, inhibition of factor Xa has appeared as an attractive principle of action for the development of new anticoagulants.[1−3] The aim of this study was to reveal new chemical scaffolds with putative inhibitory potency on factor Xa inhibitors.

METHODS

During the past decade the pharmacophore modeling approach using the software CATALYST was applied successfully in different areas of therapeutical interest as reviewed in recent publications.[4−7] The description of important chemical features in the shape of a pharmacophore model offers the advantage of a fast and reliable technique when the input data is of high quality (high affinity ligands, high resolution of the X-ray complex structure). In this study we apply a structure-based and a ligand-based approach with different aims. On one hand the structure-based pharmacophore modeling approach is able to present the interactions of a ligand to the target protein in a very specific way. Hence, selective and specific pharmacophore models will result from this application. On the other hand the ligand-based pharmacophore modeling approach is not restricted to the bound conformation of the ligand in the crystalline complex. Therefore we try to include the principal common features of various ligands and respect the flexibility of the ligand and also the flexibility of the protein in a certain extent. The ligand-based approach is able to reveal the common demand of multiple ligands and results in more general pharmacophore models that can be useful for virtual screening of large databases.

Structure-Based Pharmacophore Modeling. A crystalline complex with a ligand bound to a protein's active site is sufficient information to start the construction of a structure-based pharmacophore model. Two software tools were used in the elaboration of crucial pharmacophore patterns: The software LIGANDSCOUT[8,9] is a program for ligand interpretation and data mining in the Brookhaven Data Bank (PDB).[10] The performance of this program allows the detection of relevant interaction points between ligand and

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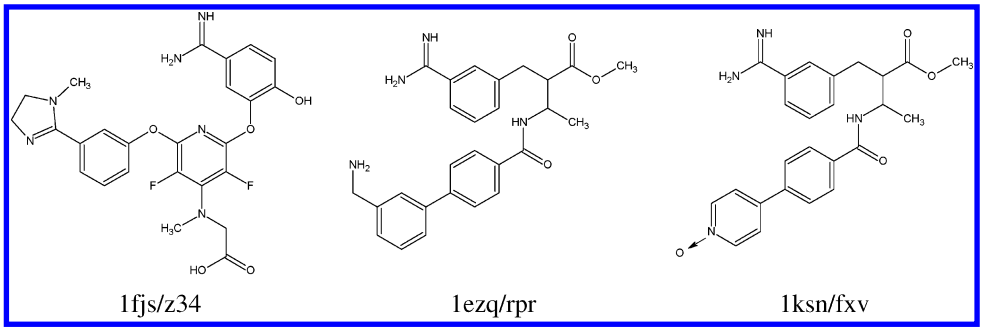


Figure 1. Highly active factor Xa inhibitors from PDB entries 1fjs, 1ezq, and 1ksn. The pdb code and ligand code is reported for each chemical structure.

protein. The binding mode of the ligand in the active site of a protein can be visualized in a sophisticated way. LIGANDSCOUT's algorithms perform a stepwise interpretation of the ligand molecules: planar ring detection, assignment of functional group patterns, determination of the hybridization state, and finally the assignment of Kekulé pattern. The interpretation of the ligand molecules is the basis for the next step, an automated generation of pharmacophore models, derived from the data provided by a crystalline complex of the PDB. An automatic detection and classification of protein−ligand interactions into hydrogen bonds, charge transfer, and lipophilic regions leads to a collection of chemical features in a pharmacophore model. The graphical user-interface can provide an integrated view of protein, ligand, pharmacophore model, and interaction lines. In our study, LIGANDSCOUT was used for the detection and interpretation of crucial interaction patterns between ligand and protein. In the second step, the software CATALYST was used for the construction of the pharmacophore models in a format that allows rapid virtual screening of multiconformational three-dimensional structure databases. The information for the pharmacophore pattern (i.e. 3D coordinates of interaction points) was obtained by the interpretation of LIGANDSCOUT pharmacophore definitions and resulted in specific interaction models that were able to map the ligand in their bioactive conformation. Protein−ligand interactions are identified by LIGANDSCOUT and pharmacophore models are exported that can be translated by a script into CATALYST pharmacophore files which are further used as queries for database searches. The structures of the three active factor Xa inhibitors used in the structure-based pharmacophore generation are shown in Figure 1.

Ligand-Based Pharmacophore Modeling. For many proteins of the human body there is still no three-dimensional structure available. The ligand-based pharmacophore approach allows the activity prediction of molecules by means of common chemical features even if the bioactive binding conformation is not known. Within the CATALYST modeling program, two possible procedures can be applied for automated ligand-based pharmacophore generation. The first algorithm is termed HypoGen and represents an activity-based alignment derived from a collection of conformational models of compounds ranging activity values of at least 4 orders of magnitude. Detailed criteria for training set selection have been presented in a previous work.[11] The second algorithm in 3D pharmacophore generation within CATALYST is called HipHop and is based on alignment of common features present in highly potent compounds. The scalar affinity values of the molecules are not regarded in this model generation mode. HipHop pharmacophore models are derived by comparing a set of conformational models and a number of three-dimensional configurations of chemical features shared among the training set molecules. Compounds of the training set may or may not fit all features of each resulting hypothesis, depending on the setting for the parameters Maximum Omitted Features, Misses, and Complete Misses. The obtained pharmacophore models are expected to discriminate between active and inactive compounds. In this work we used the common-feature based approach that is implemented in the HipHop algorithm. Only compounds with validated biological activity were considered to reveal the common chemical function of factor Xa inhibitors.

Virtual Screening Experiment of Commercial Molecular Databases. Virtual screening has established as a fast and accurate technique to obtain new compounds with desired activity profiles.[7] A screening experiment was carried out within several databases (WDI and ensemble database) of commercial compound providers in order to gain new compounds with putative activity against factor Xa. 36 active factor Xa inhibitors in preclinical or clinical development phase were extracted from the ensemble database[12] and used as a test set. This database was screened to validate the selectivity of the pharmacophore hypotheses. In contrast, the WDI search was carried out to show the restrictivity of the generated pharmacophore models rather than to identify new compounds with activity against factor Xa. A list of the database search results is given in Tables 1 and 3. All molecular structures had previously been converted into the CATALYST multiconformational data format (FAST, max 250 conformers per compound).

The elaborated pharmacophore models can be used for virtual screening of the multiconformational databases by applying two distinct algorithms, the so-called FAST or BEST database search procedures. The FAST Flexible Search command considers only already existing conformers within the database, whereas the BEST Flexible Search command additionally optimizes the conformational models during computation. A molecule must fit all the features of a CATALYST query to be retrieved as a hit. Resulting hit lists can be further ranked according to their fit values that represent the mapping quality of chemical substructures onto feature constraints as well as the distance deviation of the chemical functions from the center of the features. Therefore, the geometric fit value points out how exact the function is localized in the center of a feature sphere. A higher fit value reflects a better fit of the molecule to the pharmacophore

Table 1. Database Search Results of the Structure-Based Pharmacophore Models

		WDI DB (48405 bioactive molecules)		ensemble actives DB (36 active factor Xa inhibitors)		
pharmacophore model	chemical features	# hits	% hits	# false negatives	# actives	% actives
Hypo1−1FJS	AAAAHHP	16	0.03	30	6	17
Hypo3−1FJS	AAAAHHDD	46	0.09	32	4	11
Hypo2−1KSN	AAAHHDDD	392	0.81	26	10	28
Hypo4−1KSN	AAAHHDP	181	0.37	24	12	33
Hypo41KSNmod	AAAHHP	2953	6.10	6	28	78
Hypo3−1EZQ	AAAHHHDDDD	63	0.13	31	5	14
Hypo4−1EZQ	AAAHHHDDP	21	0.04	31	5	14

and will rank the compound on a high position in the hit list.

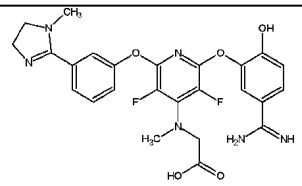
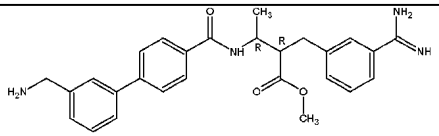
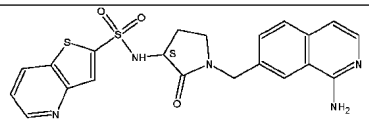
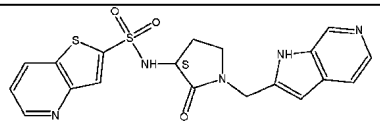
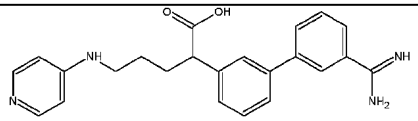
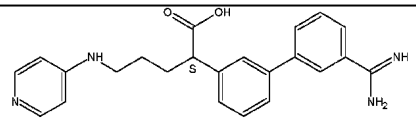
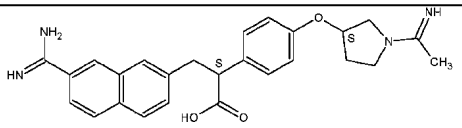
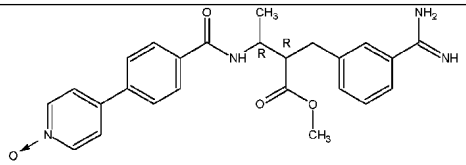
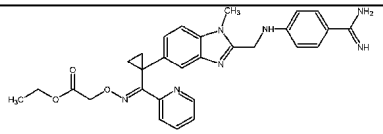
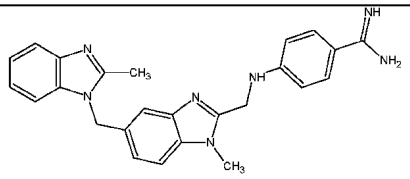
Focused Virtual Library Generation. The virtual library space can expand the virtual screening possibilities, considering the large number and broad diversity of compounds in the databases. The criteria for useful virtual libraries include high extent of structural diversity, high degree of drug-likeness/lead-likeness, and advantageous ADMET (absorption, distribution, metabolism, elimination, and toxicity) properties, and eventually synthetic accessibility. Several methods have been developed to gain the desired properties as recently discussed in a review.[7] We used a fragment-based method for rapid generation of a large virtual library with high drug-likeness. The computations' background of the software ILIB DIVERSE has been detailed out elsewhere.[13] The software package allows the user to run fragment-based generation of virtual libraries in a user-friendly interface. ILIB DIVERSE generates libraries of drug-like organic molecules for rational lead structure discovery. Compounds can be built by user-defined fragments or starting from the predefined fragment set included in the software. Several user-adjustable constraints allow a combinatorial approach providing good structural diversity as well as automated lead structure modification. The implemented filter options permit a restriction to desired properties including the minimum and maximum occurrence of chemical features, molecular weight ranges, clogP, topological polar surface area, number of rotatable bonds, number of rings, and occurrence of specific atom types. Synthetic accessibility is considered by the setting of reactivity filters after the generation process in the resulting compounds.[14] The classification of fragments into flasks and the combination of fragments from defined groups together with the weight distribution and reactivity setup of single fragments provide a rapid generation of a combinatorial library that exhibit privileged motives in the generated molecules. The fragment and flask settings are available on request by the authors.

Virtual Screening Experiment of the Focused Library. In silico screening is not restricted to databases of existing compounds but can be performed with virtual libraries as well. This approach offers the possibility to discriminate between active and inactive candidates for lead structure discovery, synthesis, and further lead optimization. We applied this method of searching for interesting candidates with desired biological activity in combination with the previous described technique of virtual library design. The focused factor Xa inhibitor library was generated from a predefined fragment set and resulted in 1000 new compounds with similar functional and spatial properties as shown by already known compounds. The technique to use well-known structural scaffolds for the generation of compounds modulating a distinct pharmacological target can be called the privileged motive approach, as reported recently.[15]

RESULTS

Pharmacophore Model of Complex 1fjs. The pdb file 1fjs contains a complex consisting of the ligand zk-807834 (pdb ligand code Z34, K_i value of 0.11 nM against factor Xa) bound to factor Xa determined at a resolution of 1.92 Å.[16] The software LIGANDSCOUT presents the interactions between the protein and the ligand and some excluded volume spheres (Figure 2). In this case three hydrophobic groups, four hydrogen bond acceptors, two hydrogen bond donors, one positive ionizable feature, and four excluded volume spheres are shown. The hydrophobic groups are aromatic rings, whereas one aromatic ring, a pyridine moiety, carries five substituents, among them two fluoro atoms, one tertiary amine, and two ether functions. The hydrophobic feature contains following definitions, as described in the CATALYST tutorial: a continuous set of atoms that are not adjacent to any concentration of charge (charged atoms or electronegative atoms), so that the atoms have surface accessibility, including phenyl, cycloalkyl, isopropyl, and methyl.[17] Therefore, this ring cannot be recognized as a hydrophobic region by CATALYST. The positive ionizable feature and the two hydrogen bond donors characterize the amidine group bound to an aromatic ring. CATALYST is not able to handle two different kinds of features at one point in the three-dimensional space. So, two hypotheses were built manually to describe the pharmacophore correctly. The first hypothesis (Hypo1−1FJS) contains two hydrophobic rings, four hydrogen bond acceptors with projected points, and one positive ionizable group. The excluded volume spheres were omitted due to selectivity reasons (the pharmacophore already consists of seven features and forms a very specific filter unit). The second hypothesis (Hypo3−1FJS) differs from the first one only at the position of the amidine group. Instead of one positive ionizable group, two hydrogen bond donors were chosen. Thus the second pharmacophore model includes eight features (two hydrophobic groups, four hydrogen bond acceptors with projected points, and two hydrogen bond donors without projected points). Figure 2 shows Hypo1−1FJS and Hypo3−1FJS, respectively. Searching of the Derwent WDI database with these two pharmacophore models results in 16 (Hypo1−1FJS) and 46 (Hypo3−1FJS) hits, respectively. This represents 0.03% and 0.09% of the database, respectively. With Hypo1−1FJS six and with Hypo3−1FJS four active factor Xa inhibitors within preclinical or clinical development phase were retrieved (Figure 3).[12]

Table 2. Chemical Structures and Biological Activities of the Training Set Compounds for Common Feature Based Pharmacophore Models

PDB Entry	Chemical Structure	Activity Ki (IC_{50}*) [nM]	Resolution [Å]	Reference
1FJS		0.2	1.92	Adler et al.[20]
1EZQ		0.9	2.20	Adler et al.[20]
1F0R		22	2.10	Adler et al.[20]
1F0S		18	2.10	Adler et al.[20]
1XKA		272*	2.30	Kamata et al.[21]
1XKB		ND	2.40	Kamata et al.[21]
1FAX		70*	3.00	Nagahara et al.[22]
1KSN		0.52	2.10	Chu et al.[23]
1G2L		57	1.90	Nar et al.[24]
1G2M		40	3.02	Nar et al.[24]

Pharmacophore Model of Complex 1ksn. The pdb entry 1ksn (resolution 2.1 Å) contains factor Xa complexed with the inhibitor fxv-673 (pdb ligand code FXV, K_i value of 0.4 nM against factor Xa**)**.[18] The pharmacophore generated with the LIGANDSCOUT software exhibits 11 features including three hydrophobic groups, three hydrogen bond acceptors, four hydrogen bond donors, one positive ionizable function, and six excluded volume constraints (Figure 4). Two

Table 3. Database Search Results of the Ligand-Based Pharmacophore Models

		WDI DB (48405 biological active molecules)		ensemble actives DB (36 active factor Xa inhibitors)		
pharmacophore model	chemical features	# hits	% hits	# false negatives	# actives	% actives
Hypo7confmin.08	HarHarAAA	1307	2.7	11	25	69
Hypo7confmin.05	HarHarAAD	1057	2.2	9	27	75
Hypo8confmin.01	HarHarAAD	1057	2.2	9	27	75
Hypo8confmin.02	HarHarAAA	1873	3.9	8	28	78
Hypo8confmin.02shape	HarHarAAAshape	127	0.3	23	13	36

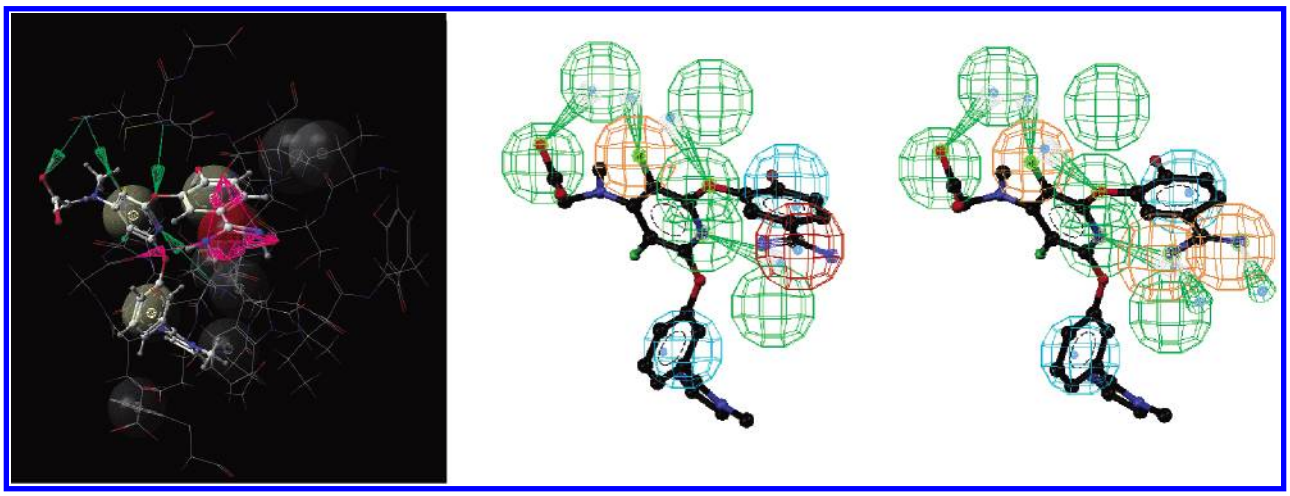


Figure 2. Pharmacophore identification and generation with LIGANDSCOUT (left) and CATALYST (right) of 1fjs (Hypo1−1FJS and Hypo3−1FJS). CATALYST color code: green − hydrogen bond acceptor, orange − hydrogen bond donor (top of both pictures), orange − hydrogen bond donor (right side in right picture), red − positive ionizable group, blue − hydrophobic region.

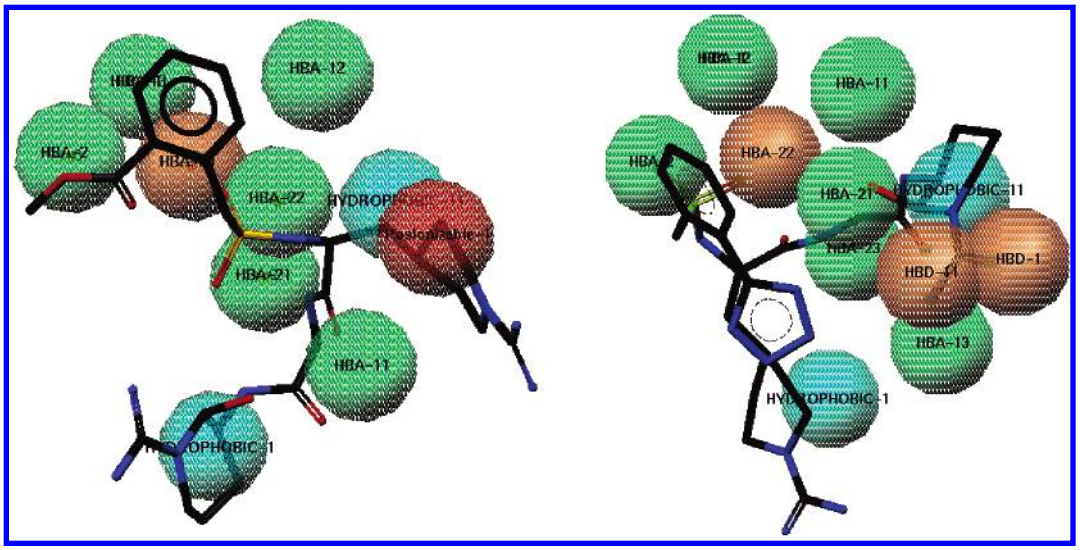


Figure 3. Active factor Xa inhibitors from the ensemble database in preclinical or clinical development mapping the pharmacophore chemical features derived from pdb complex 1fjs.

hydrogen bond donors and one positive ionizable group characterize the amidine function in this structure. Further components of the factor Xa inhibitor are three hydrophobic groups, one amide, one ester, and one hydroxylamine moiety. As CATALYST does not recognize the nitrogen of the amidine as a hydrogen bond donor, the pharmacophore model 'Hypo1−1KSN' used consists finally of eight features including three hydrogen bond donors, three hydrogen bond acceptors, and two hydrophobic groups. This hypothesis retrieved 30 (this represents 0.06%) compounds from the Derwent WDI database; however, all of them possessed a molecular weight above 800 g/mol. The aim to identify low-molecular weight compounds with this pharmacophore model was not met, and therefore simplification of this hypothesis was done which resulted in the following pharmacophore model: All projected points of the hydrogen bond donors and acceptors were omitted (Hypo2−1KSN), which resulted in 392 hits (0.81%) when executing the WDI database search. Only 23 compounds (0.05%) are characterized by a molecular weight lower than 600. Among the hitlist retrieved by the pharmacophore model Hypo2−1KSN, 10 factor Xa inhibitors in preclinical or clinical trial phase were obtained. An example is presented in Figure 5.[12]

In Hypo3−1KSN, two hydrogen bond donors were replaced by a positive ionizable function. Using Hypo3−1KSN as a search query the virtual screening of the Derwent

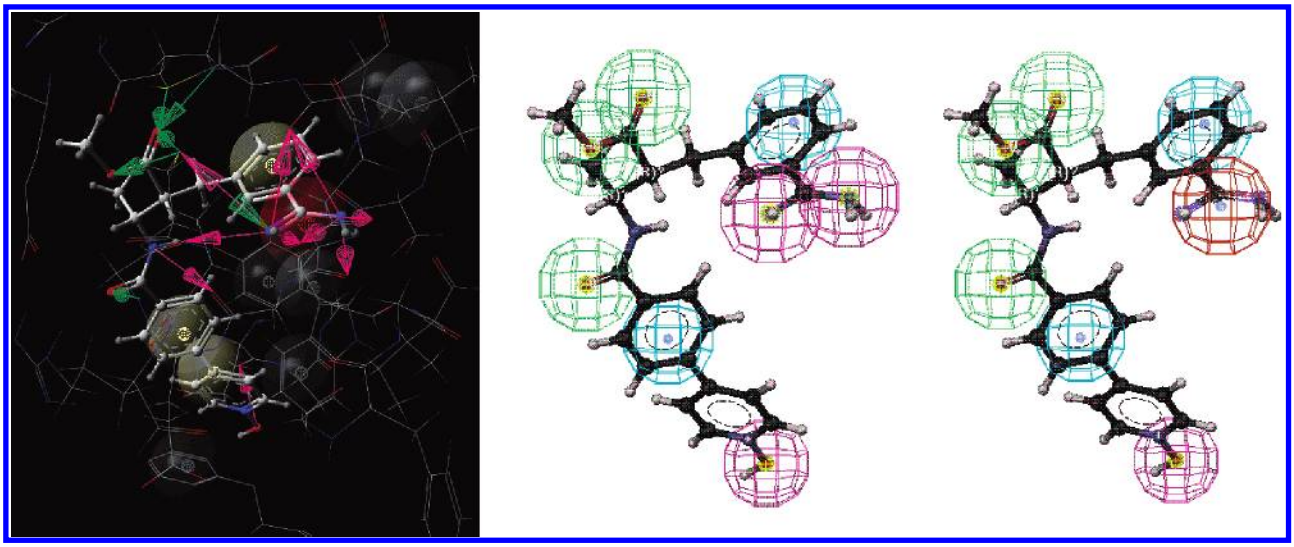


Figure 4. Pharmacophore identification and generation with LIGANDSCOUT (left) and CATALYST (right) of 1ksn. Mapping of fxv-673 on Hypo2−1KSN and Hypo4−1KSN is shown. CATALYST color code: green − hydrogen bond acceptor, magenta − hydrogen bond donor, red − positive ionizable group, blue − hydrophobic region.

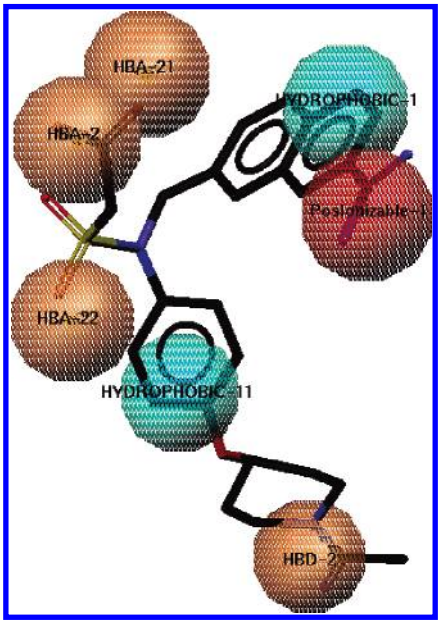


Figure 5. Active factor Xa inhibitor retrieved from the ensemble database in preclinical/clinical development mapping the pharmacophore chemical features derived from pdb complex 1ksn.

WDI database resulted in nine hits with a molecular weight above 800. After the same modification as described above concerning Hypo1−1KSN, the new Hypo4−1KSN found 181 compounds (0.37%) (141 with a molecular weight above 1000 (0.29%)) in the entire Derwent WDI database and 12 compounds in clinical or preclinical development for inhibition of factor Xa. In Figure 4 the ligand fxv-673 is mapped to Hypo2−1KSN and Hypo4−1KSN, respectively.

Pharmacophore Model of Complex 1ezq. The complex 1ezq (resolution 2.2 Å) contains the ligand rpr-128515 (pdb lig code RPR, K_i value of ligand of 0.9 nM against factor Xa) bound to factor Xa.[19] The pharmacophore model generated within the LIGANDSCOUT software contains the following interactions: three hydrophobic groups, three hydrogen bond acceptors, four hydrogen bond donors, and one positive ionizable group. Additionally, six excluded volumes are present. The first pharmacophore model built from the bound conformation of RPR within CATALYST consists of 10 features including three hydrophobic groups, three hydrogen bond acceptors without projected points, two hydrogen bond donors with projected points, and two hydrogen bond donors without projected points (Figure 6). The latter features characterize the amidine group of the compound. The projected points of the hydrogen bond acceptors were omitted because otherwise the ligand would not fit into the hypothesis. This hypothesis (Hypo1−1EZQ) detects only three compounds (0.006%) in the Derwent WDI database; therefore, all projected points of hydrogen bond donors were deleted. The WDI search with the revised hypothesis (Hypo3−1EZQ) as search query resulted in 63 hits (0.13%) and delivered five compounds in clinical or preclinical test phase (Figure 7).

To characterize the amidine function of the discussed ligand extensively, Hypo2−1EZQ was created manually. It differs from Hypo1−1EZQ only slightly: The two amidine hydrogen bond donors are replaced by a positive ionizable function. Hypo2−1EZQ includes nine chemical functions: three hydrophobic groups, three hydrogen bond acceptors without projected points, two hydrogen bond donors with projected points, and one positive ionizable group. Using Hypo2−1EZQ (not shown) as a search query, no compounds of the Derwent WDI database were detected at all. Therefore, this hypothesis was unconstrained by removing the projected points of the hydrogen bond donors. The resulting pharmacophore model (Hypo4−1EZQ) identifies 21 compounds (0.043%) of the Derwent WDI database among them five active factor Xa inhibitors.[12] Figure 6 shows the pharmacophore models Hypo3−1EZQ and Hypo4−1EZQ respectively mapped to the inhibitor rpr-128515. Figure 7 shows compounds in preclinical/clinical development matching the pharmacophore pattern.

In the *compare fit* procedure the best hypothesis regarding the capability to identify a high number of active factor Xa inhibitors (hypo4−1ksn) was modified in order to retrieve more of the active factor Xa inhibitors. This resulted in a hypothesis (Hypo4−1KSNmod) without the hydrogen bond donor in the bottom. This pharmacophore model was able to retrieve 28 of 36 (78%) active factor Xa inhibitors and

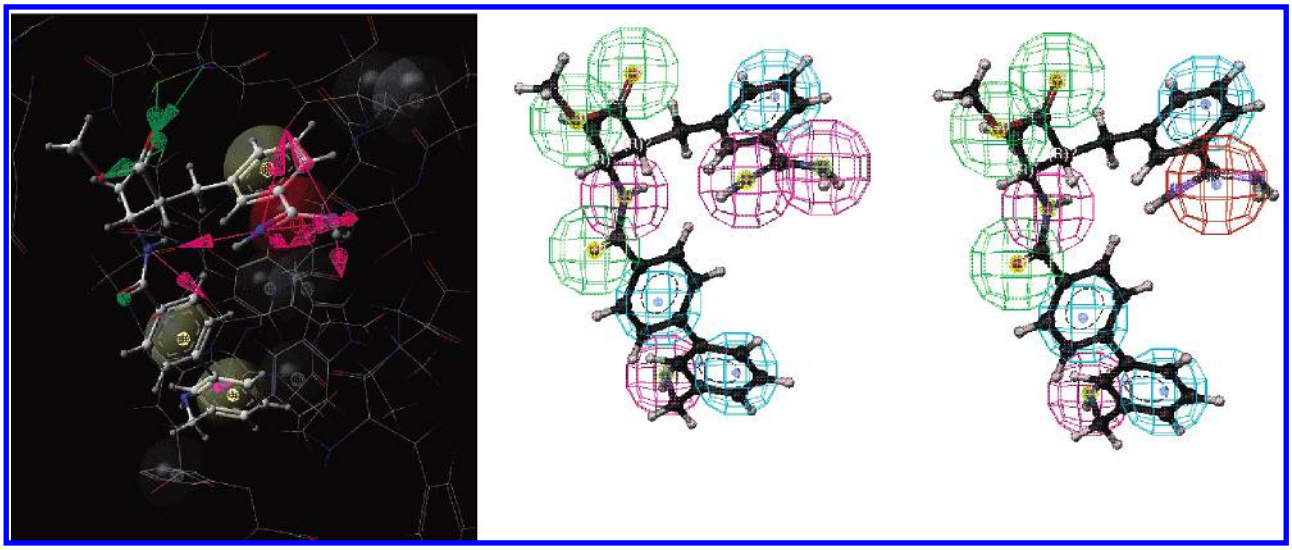


Figure 6. Pharmacophore identification and generation with LIGANDSCOUT (left) and CATALYST (right) of 1ezq. CATALYST color code: green − hydrogen bond acceptor, magenta − hydrogen bond donor, red − positive ionizable group, blue − hydrophobic region.

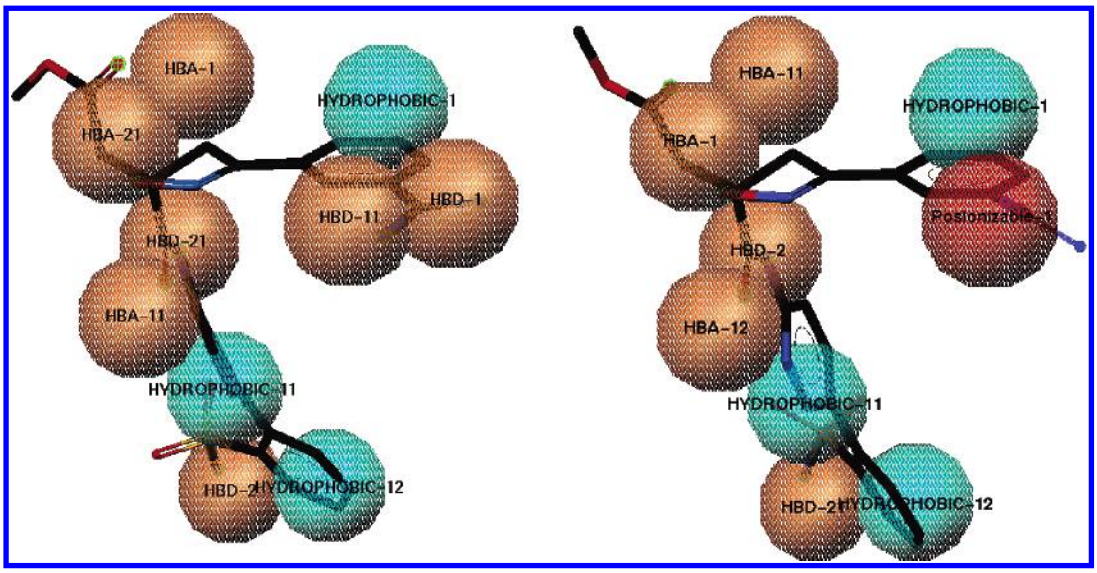


Figure 7. Two active factor Xa inhibitors from the ensemble database in preclinical or clinical development mapping the pharmacophore chemical features derived from pdb complex 1ezq.

by the way increased the hit rate in the WDI to 2953 compounds. One possible reason for nonretrieval of all active factor Xa inhibitors may be different occupancy of the active site and hence just a partial mapping of the pharmacophore model. Further, a completely different binding mode of the compounds not retrieved by the structure-based pharmacophore model Hypo4−1KSNmod is reasonable in order to explain the factor Xa activity.

The similarity of the chemical structures of the ligands 1ezq/rpr and 1ksn/fxv can be seen in Figure 1. Hence we compared the pharmacophore models hypo4−1ksn and hypo4−1ezq in the CATALYST *View Hypothesis Workbench* and identified a similar arrangement of chemical features as shown in Figure 8. A highly similar binding mode can be suggested for these two ligands.

Common Feature Based Pharmacophore Models. To compare pharmacophore models created manually from a bound conformation to automatically generated hypotheses by CATALYST, the 10 ligands from distinct pdb entries were used as a starting point to generate hypotheses with the *HipHop* algorithm. These inhibitors can be divided into high-affinity inhibitors (<1nM; 1fjs, 1ezq, and 1ksn) and low-affinity inhibitors (>10nM; 1fos, 1for, 1fax, 1xka, 1xkb, 1g2l, and 1g2m).

After energy minimization of every single molecule, a generation of conformational models for all molecules was performed. The 10 compounds, with associated conformational models, were then used for common feature hypotheses generation. The chemical features hydrogen bond donor, hydrogen bond acceptor, hydrophobic feature, and positive ionizable group were preselected for the generation process. The pharmacophore models, e.g. Hypo3confmin.01, consisted of one hydrogen bond donor and three hydrogen bond acceptors. The maximum omitted features values were set to zero for the high-affinity inhibitors and to one for the low-affinity inhibitors to obtain the presented pharmacophore model Hypo3confmin.01. Two more four-feature pharmacophore models, either containing one hydrophobic group and three hydrogen bond acceptors (Hypo4confmin.01) or two hydrogen bond donors and two hydrogen bond acceptors (Hypo4confmin.03), were generated in a more constrained generation setup: The value for the maximum omitted

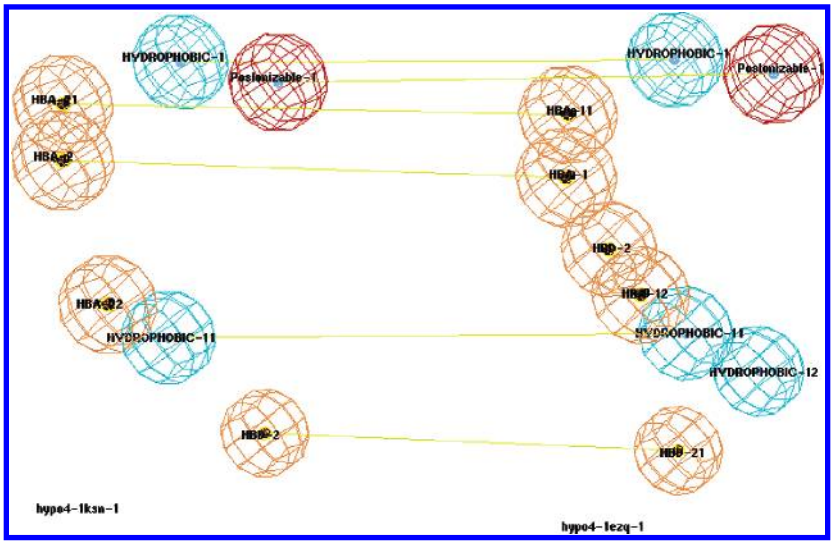


Figure 8. Comparison of the structure-based pharmacophore models hypo4−1ksn and hypo4−1ezq in CATALYST. Corresponding features can be identified and suggest a similar binding mode in the active site of factor Xa.

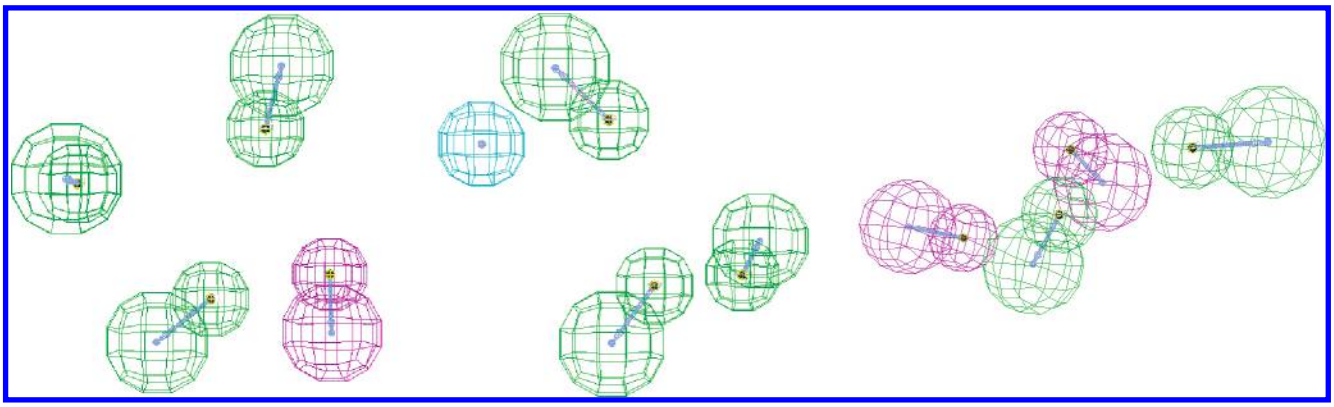


Figure 9. Common feature CATALYST pharmacophores (Hypo3confmin.01, Hypo4confmin.01, and Hypo4confmin.03) generated from 10 ligands, each represented by a multiconformational model.

features were set to zero for all 10 training set compounds. Thus every single member of the training set collection had to fit every chemical feature of the pharmacophore models (Figure 9).

The generated pharmacophore models represent the common chemical features of both, highly active and low active factor Xa inhibitors, regarding the conformational models of 10 ligands from crystalline complexes. Because of the inclusion of the low-affinity inhibitors the in silico screening results of three-dimensional structure databases was not restrictive (i.e. 20% of Derwent WDI were retrieved), as expected by us. Therefore these models are useful for initial screening of virtual libraries, but an enhancement concerning the specificity of the pharmacophore pattern is necessary to create high quality pharmacophore models.

In a next step a pharmacophore model was gained from the bound, bioactive conformation of every ligand. In fact, only 10 conformers (one conformer per ligand) were therefore used in hypothesis generation. Hypo3noconf.01 includes two hydrophobic groups and two hydrogen bond acceptors (Figure 10). In the virtual screening procedure these pharmacophore models revealed too many molecules to be considered as selective and representative.

To specify the crucial interaction points between ligand and protein the general hydrophobic chemical feature was replaced by the hydrophobic aromatic function. CATALYST

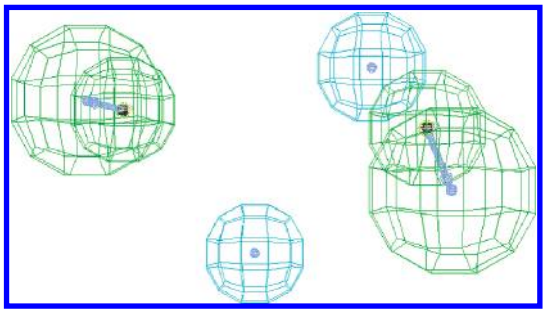


Figure 10. Common feature CATALYST pharmacophores generated from 10 ligands, each represented by a single bioactive conformation. Hypo3noconf.01 containing two hydrophobic groups (blue) and two hydrogen bond acceptors (green).

was able to generate pharmacophore models consisting of five features, when using the conformational models of the 10 ligands as input. Hypo7confmin.08 and Hypo8confmin.02, both including two hydrophobic aromatic ring features and three hydrogen bond acceptors, detected less than 1307 and 1873 compounds (2.7% and 3.9%) in the Derwent WDI database. Hypo7confmin.05 and Hypo8confmin.01, both containing two hydrophobic aromatic groups, one hydrogen bond donor and two hydrogen bond acceptors, found 1057 compounds (2.2%) in Derwent WDI database search (Figures 11 and 12).

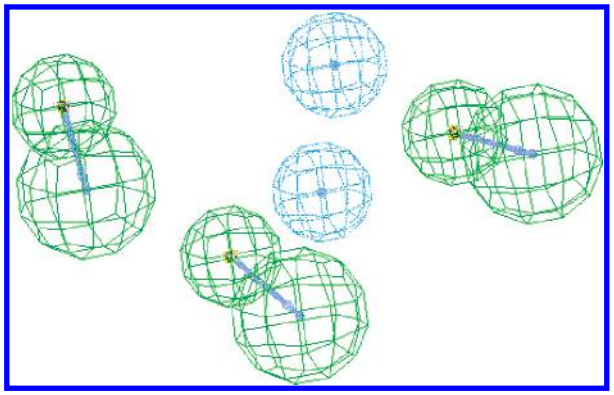


Figure 11. Hypo7confmin.08 common feature CATALYST pharmacophore generated from 10 ligands, each represented by a multiconformational model. The hydrophobic feature was replaced by a hydrophobic aromatic feature to enhance the selectivity. CATALYST color code: green − hydrogen bond acceptor, magenta − hydrogen bond donor, blue − hydrophobic aromatic.

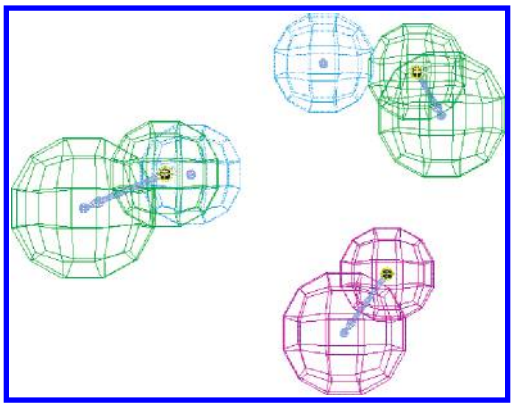


Figure 12. Hypo8confmin.01 common feature CATALYST pharmacophore generated from 10 ligands, each represented by a multiconformational model. The hydrophobic feature was replaced by a hydrophobic aromatic feature to enhance the selectivity. CATALYST color code: green − hydrogen bond acceptor, magenta − hydrogen bond donor, blue − hydrophobic aromatic.

Using just the bound conformations of the 10 factor Xa inhibitors, CATALYST generated pharmacophore hypotheses with four features, when the maximum omitted features of the low-affinity inhibitors were kept at one. Setting the maximum omitted features to zero for all ligands, the hypothesis generation process resulted in pharmacophore models with just three features. All hypotheses automatically generated by CATALYST using the HipHop algorithm were able to detect at least 25 (69%) of the 36 compounds in the ensemble test-subset of active factor Xa inhibitors in preclinical or clinical development phase (Table 2).[12]

To generate a highly restrictive and selective ligand-based pharmacophore model the shape constraints of the three bioactive conformations of the three ligands shown in Figure 1 (factor Xa inhibitors with biological affinity values in the subnanomolar range) are combined into one hypothesis termed Hypo8confmin.02shape (Figure 13). In virtual screening experiments, out of the WDI 127 compounds (0.3%) and 14 active factor Xa inhibitors (39%) of the ensemble database were retrieved. The generated ligand-based pharmacophore model Hypo8confmin.02shape is highly selective and contains the common features and shape constrains between the factor Xa inhibitors. This hypothesis has practical utility in the initial screening of large databases, because it can rapidly

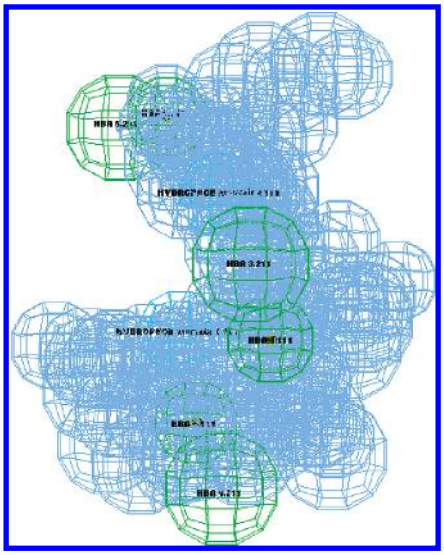


Figure 13. Hypo8confmin.02shape is highly selective and contains the common features and shape constraints between the factor Xa inhibitors.

and accurately reduce the number of candidates active against the target of interest. Other virtual screening techniques, e.g. ligand docking, can afterward be applied for a more detailed examination.

The second automated pharmacophore generation algorithm within the software CATALYST is Hypogen. A homogeneous training set including high selective and diverse compounds with activity values spanning at least 4 orders of magnitude (all derived from a comparable test system) is required for a valid quantitative predictive pharmacophore model. The biological systems used for the determination of the inhibition of factor Xa vary among the literature. Therefore it was not possible to collect a suitable number of members of the training set (at least 16).

Hits from the Virtual Screening Procedure 1. In Figure 14, some of the compounds that are known as active factor Xa inhibitors and were retrieved by the constructed pharmacophore models are presented.

Focused Virtual Library Generation Setting in ILIB DIVERSE. Besides the numerous recently published work dealing with the rational design of factor Xa inhibitors, several entries of human factor Xa inhibitors are deposited in the Protein Data Bank.[10] Three of the PDB Entries (1ksn, 1ezq, and 1fjs) are shown and used to guide our virtual library generation process. The ligands show affinity data (K_i values) in the subnanomolar range against human factor Xa. The structural templates of the three ligands from the corresponding PDB entries are presented in Figure 1.[16,18,19]

Based on the structure of the factor Xa inhibitor templates, we defined several flasks for the library generation process (Figure 15). A flask contains selected or predefined chemical scaffolds and is used for the combinatorial generation of the virtual libraries. An overview of the flask content is presented in Table 4.

To start at a hydrophobic part (flask 1) of the molecules, benzene and phenol (benzenes group), naphthalene (carbocycles group), and several selected heterocycles (indol, thiophen, coumaron, benzisoxazole) were included in flask 1. The reactivity was increased to value 6 and 4 in position 1 and 3 of the benzene and position 2 and 4 of the phenol

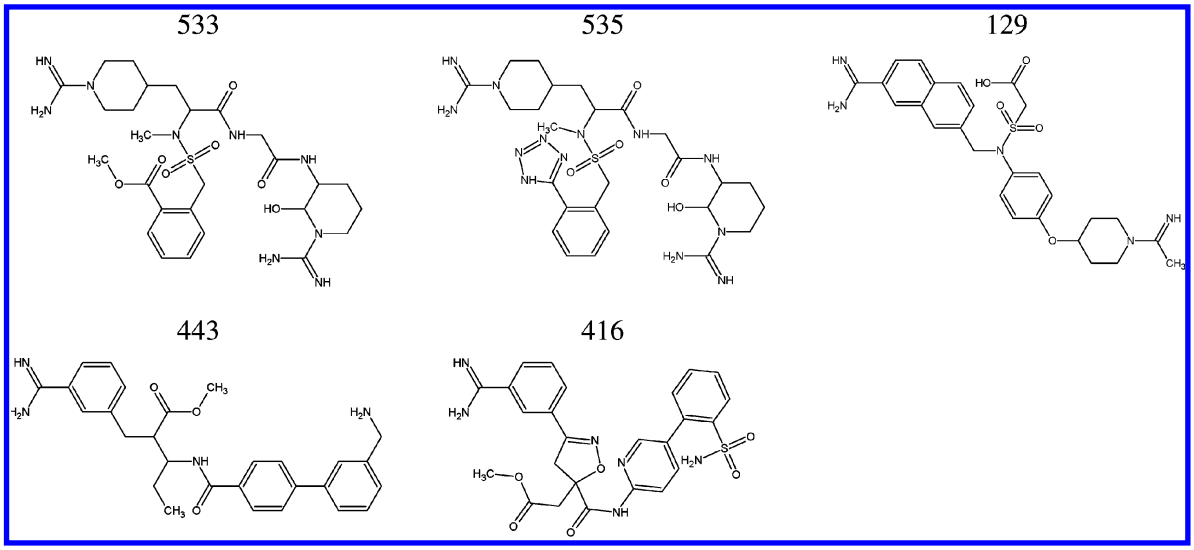


Figure 14. Known factor Xa inhibitors that were retrieved from the ensemble database by the presented pharmacophore models.[14]

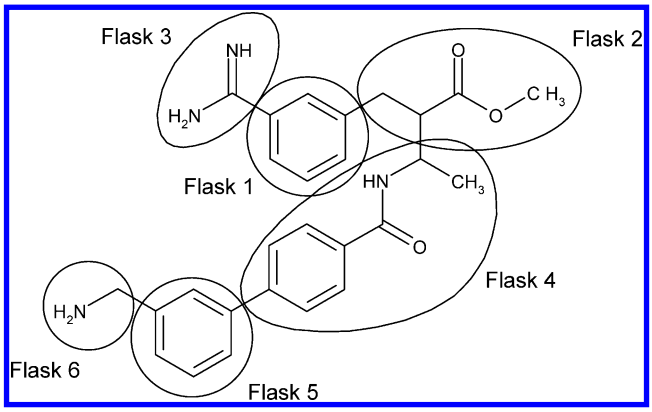


Figure 15. Fragmentation of factor Xa inhibitors into flasks containing building blocks used for compound generation within ILIB DIVERSE.

moiety. Further, the substitution was omitted on the rest of the cyclic carbon atoms to ensure the desired structural pattern. The reactivity of the other fragments was assigned in a similar mode. The modified reactivity settings are shown in Figure 16. A weight of 100% was set on the benzene fragment and weight of 25% was set to all other fragments of flask 1. The ester and alcohol groups form the second flask. We restricted the included esters to ethyl acetate, methyl acetate, and methyl acetoacetate. The following modified reactivity settings were applied: The terminal carbon atoms were set to higher activity value, e.g. 3 and the flag "Reset reactivity after first substitution" was set to 'on' for each atom with increased activity. Hence, the multiple substitutions on one carbon atom are reduced significantly. An example is presented in Figure 16. The formamidine moiety of flask 3 is selected to substitute flask 1 in one of the earlier defined positions. A modification of the reactivity parameter was applied and is presented in Figure 16. Furthermore, the "Reset reactivity after first substitution" flag was selected in order to omit a multiple substitution of the carbon atom of the formamidine fragment. All selected fragments for flask 4 are derived from the carbocycles group, the bicyclic heterocylces group, and the aliphatics group. The modified reactivity values were kept to lead to chain-shaped molecules and prevent a high degree of substitutions on single fragment parts. The "Reset reactivity after first substitution" flag was set to 'on' for this reason as well. The next hydrophobic part of the molecules was presented by a selection of different structural moieties in flask 5. Besides the benzene fragment, other entries from the carbocycles group, the monocyclic heterocycles group, aliphatics group, and the bicyclic heterocycles group were chosen. Another fragment used was the sulfonamide moiety, a substructure that is found in several factor Xa inhibitors.[25] The settings for flask 6 were selected to extend the length of the chain within the direction of the fragments that were used on flasks 4 and 5. The monocyclic and bicyclic heterocycles groups were chosen besides the amines group. The final building block for the focused library, flask 7 (not shown in Figure 15) consisted of a selection of aliphatic moieties, like methane and ethane. The reactivity values are kept the same as in flask 4.

The fragment selections for flask 4−7 were considered to find new scaffolds with similar chemical and spatial demands as active factor Xa inhibitors. The generated molecules were desired to be not too similar in their topological properties to stretch the chemical and structural variability of the compounds in the generated focused virtual library. The weight settings were kept as defined in the default parameter of every single compound.

Recommended Filter Settings. Application of Lipinski's 'rule of five' filter ensures high drug-likeness and oral bioavailability of the generated compounds.[26] The stereochemistry setting was defined to eliminate all compounds with a stereocenter (R/S). This restriction was used to show that a library can be generated without a single chiral molecule in order to find compounds that are (i) possibly more easy to synthesize and (ii) avoid all problems related with stereospecific determination of biological effects. This generation process results in a drug-like library of compounds that share the similar shape and functions as known factor Xa inhibitors. On standard hardware, time consumption for each generated compound came up to 137 ms. The time per included library molecule (excl. filtered compounds) came up to 2525 ms. This time expense corresponds to the highly restrictive filter settings in order to find only achiral substances. The library generation efficiency can be enhanced

Table 4. Flask Content Used for Fragment Based Focused Library Generation

flask 1	flask 2	flask 3	flask 4	flask 5	flask 6	flask 7
benzene	ethyl acetate	formamidine	cyclohexane	benzene	1,3-thiazole	aliphatics
benzene	methyl acetate		indene	naphthalene	furan	
naphthalene	methyl acetoacetate		naphthalene	cyclohexane	imidazole	
indole	ethanol		ethane	indene	indolizine	
thiophene	methanol		methane	1,3-thiazole	indolizine	
coumaron			benzisoxazole	furan	isothiazole	
benzisoxazole			coumaron	imidazole	oxazole	
			indole	indolizine	pyrazole	
			isoquinoline	isothiazole	pyridine	
			aliphatics	oxazole	pyrimidine	
				pyridine	pyrrole	
				pyrazole	pyrrolidine	
				pyrimidine	thiophene	
				pyrrole	benzisoxazole	
				pyrrolidine	coumaron	
				thiophene	indole	
				ethane	isoquinoline	
				methane	butlyamine	
				benzisoxazole	colamine	
				coumaron	diethylamine	
				indole	dimethylamine	
				isoquinoline	ethylamine	
				sulfonamide	methylamine	
					morpholine	
					piperazine	
					piperidine	

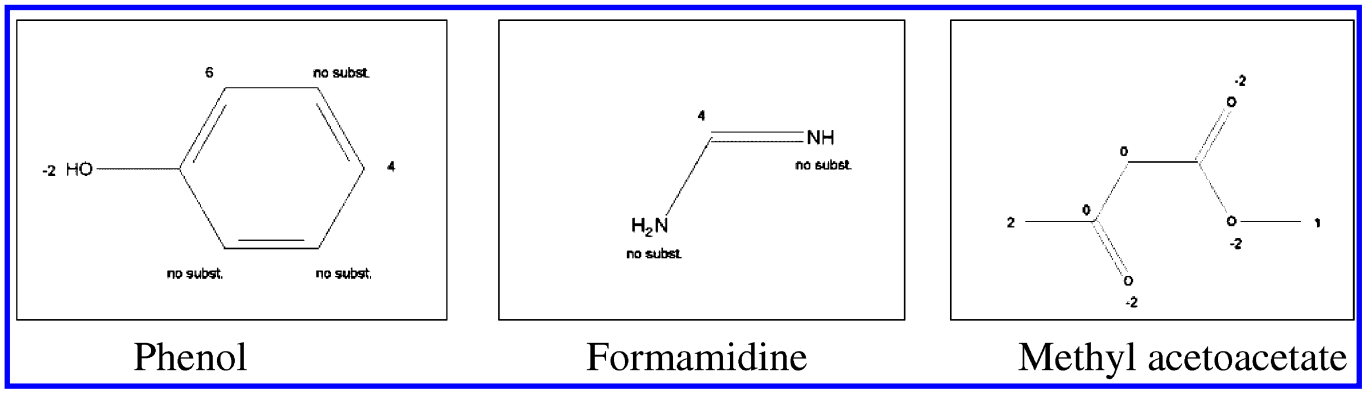


Figure 16. Examples of reactivity settings for fragments used in the generation process.

by the selection of different fragments and/or fragment weights, which do not contradict filter definitions. In our application case, 95% of the molecules were discarded during the filtering process. About 55% of the filtered molecules mismatched the stereo constraint, and about 27% of the molecules exceeded the desired estimated logP value of max. 5.0. The remaining 5% of the generated chemical structures represent the focused virtual library (1000 compounds) containing the diverse, drug-like molecules exhibiting the desired properties. Conclusively, a library generation experiment within a reasonable time frame (35 min) is even possible when extremely restrictive filter criteria are applied. Examples of generated compounds are given in Figure 17. The Report Section is available on request by the authors and provides information on the generation process as well as on the estimated clogP and molecular weight distribution of the virtual library.

Hits from the Virtual Screening Procedure 2. We performed a pharmacophore search in the generated library to ensure the functional and spatial properties of the virtually generated compounds. Some hits are shown in Figure 17. The resulting chemical structures represent de novo derived molecules with high drug-likeness and a good chance to show the desired biological activity, actually the inhibition of factor Xa.

DISCUSSION

Lead structure identification is a topic that can be addressed by different ways and methods. The pharmacophore modeling approach presents itself as a detailed description of favorable chemical properties of a specific substance group binding to a pharmacologically interesting target. To describe the interactions of an inhibitor to a protein, coordinates are transferred from a pdb file into a pharmacophore model in order to represent the predominant interactions. Several considerations must be regarded in the generation of a pharmacophore model: On one hand, a pharmacophore model tends to become too specific when all interaction points are required. This happens easily by the rigorous construction of a structure-based pharmacophore model. In consequence, a database search is not feasible, and the number of features must be reduced, e.g. projected points of vectorized features can be eliminated. This enables us to find other factor Xa inhibitors but prevents us from

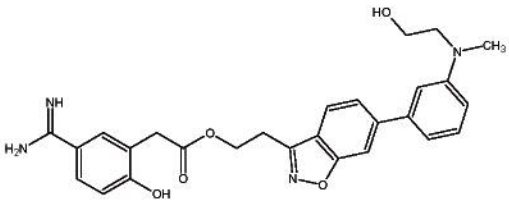
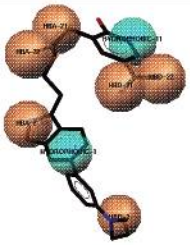
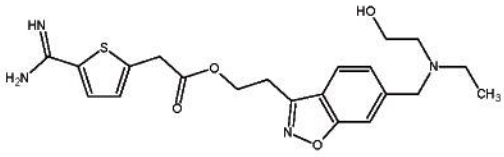
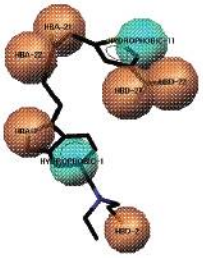
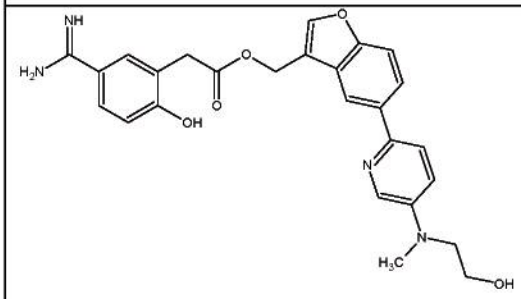
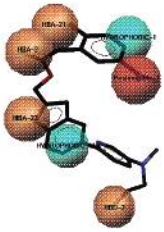
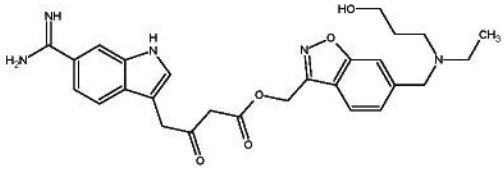
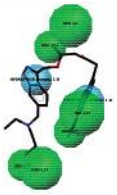
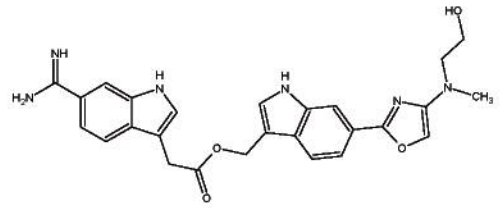
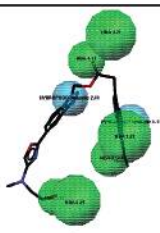
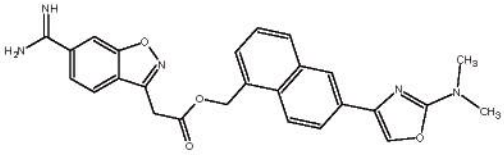
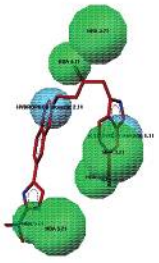
2D Structure	3D Mapping	Mapped Hypothesis	Mapped Molecule	**Fit Value**
		Hypo2-1KSN	631	**5.77**
		Hypo2-1KSN	349	**5.20**
		Hypo4-1KSN	883	**4.86**
		Hypo7confmin.08	285	**3.58**
		Hypo7confmin.08	396	**3.94**
		Hypo7confmin.08	253	**3.45**

Figure 17. Examples of generated structures with new scaffolds and similar chemical and spatial demands as active factor Xa inhibitors.

depicting the direct interaction with the protein chains. The content of the regarded databases is of main interest, considering the structural diversity and drug-likeness of the included molecules. On the other hand, pharmacophore models can be used, as shown in this study, to describe the interactions that are common among several highly active compounds. This approach is used in the ligand-based approach as shown before. Such pharmacophore patterns show a more general view of the interaction patterns. The primary screening of compounds can be performed with pharmacophore models that present a broad filter. Ligand-based pharmacophore models are often less specific than the structure-based models but may be useful for the detection of new scaffolds, considering the structural diversity. A problem that can hardly be addressed in the pharmacophore virtual screening procedure is the filtering of compounds according to favorable pharmacokinetic properties. As in commercial molecular databases often compounds are present that do not obey important rules for e.g. oral bioavailability (Lipinski's rule of five), subsequent filtering has to be performed. This separate time-consuming step has to be performed either once before screening the database or each time when analyzing the hit lists.[26] In our ILIB DIVERSE approach, this filtering process is already included in the generation phase of virtual compound libraries. This program has a variety of chemical filters as maximum and minimum number of expected chemical features, molecular weight ranges, clogP, rotatable bonds, number of rings, and many more. The output of molecules corresponds to the user's predefined parameter setting and enables the medicinal chemist to include ideas and demands in the generation process. Because of the fact that CATALYST cannot place two chemical features onto one structural element, several complementary pharmacophore models were generated and used in combination to describe the binding site. We try to point out that our study describes not just another example of a successful application of pharmacophore queries to find actives in virtual screening or drug-like databases but a novel strategy to combine the structures of a focused virtual library and the three-dimensional chemical demands of pharmacophore models to identify novel lead structure candidates. We compare different ways of pharmacophore generation in the two software programs CATALYST and LIGANDSCOUT: The sensitivity of pharmacophore searches is influenced by the feature definition and the algorithm used in the proceeding of the pharmacophore construction. The structure-based pharmacophore models are highly restrictive and selective in the database queries, whereas the ligand-based common feature pharmacophore models are useful in the initial screening to reduce the number of candidate hits active against a target. Automated detection of structure-based pharmacophore models (LIGANDSCOUT) vs automatically constructed ligand-based pharmacophore models (CATALYST) were submitted in the study. Doubtless the structure-based design is a more accurate method compared to the ligand-based approach concerning the starting information (spatial extension and corresponding chemical features in the active site) of the binding pocket of a protein, but it lacks the conformational flexibility of the ligand. This issue is addressed in the ligand-based approach of CATALYST. The bioactive conformation of the ligand bound in the pocket derived from a crystalline complex is a high qualitative information for the molecular modeler to develop a hypothesis that describes the necessary spatial and functional demands of putative modulators of the binding pocket.

Another point of view in the pharmacophoric description of the binding pocket is that a number of pharmacophore models are needed in CATALYST to fully describe one binding pocket due to the limitation of the program that only allows the user to set one single chemical feature onto one specific chemical group or atom.

An innovative method in LIGANDSCOUT is the overlaying of 3D pharmacophores which has been presented recently.[27] It can be used to select and combine chemical features from structure-based pharmacophores, such that a new model is discovered that is significantly broader in its application scope and therefore allows the identification of new lead structures for a specific target. This method will be issue of further investigations to find common chemical features in structure-based approaches and by the way implementing a completely new application of 3D crystalline structure information in structure-based design.

CONCLUSION

A combination of two different approaches was applied in this study to get an insight into the biochemical properties eventually needed for the inhibition of factor Xa inhibitors. First, pharmacophore models were generated from highly active human factor Xa inhibitors (activity ranges in subnanomolar range) in two ways, a structure-based as well as a ligand-based approach. This technique of pharmacophore modeling reveals crucial chemical functionality necessary for selective interaction of factor Xa and ligands with inhibitory potency. We used two software packages, CATALYST and LIGANDSCOUT, for the identification and generation of pharmacophore models. Second, a fragment-based virtual library was generated by means of the innovative software tool ILIB DIVERSE. The user modifiable parameters made it possible to generate a library that yielded chemical scaffolds with putative inhibitory potency against factor Xa. The combination of the pharmacophore modeling and the virtual combinatorial library shows up a new approach to get clues about novel structural patterns with similar spatial and functional properties as known factor Xa inhibitors. We present the de novo derived compounds to guide rational drug design and enable the discovery of novel lead structures. A further validation of the suggested structures needs chemical synthesis and biological testing to ensure the quality of the results. We plan to test several new scaffolds from commercial molecular databases to validate our hypothesis before the synthesis is started for one of these compounds.

ACKNOWLEDGMENT

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