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Confirmation of Predicted Activity for Factor XIa Inhibitors from a Virtual Screening Approach

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Significance

High-throughput screening approaches, where hundreds of thousands of compounds are evaluated in microamounts for their activity against certain targets, can regularly result in hit rates that are only a fraction of a percent. Here, we take a previously developed machine-learning classification model (with the Signature molecular descriptor) used to identify active compounds against Factor XIa and experimentally verify the virtual screening model predictions. Of 21 predicted compounds tested, seven show activity against Factor XIa, a 33% hit rate. © 2014 American Institute of Chemical Engineers *AIChE J*, 60: 2741–2746, 2014

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PubChem is a database of molecules maintained by the National Institutes of Health. Among other information, the database contains biological activity data for about 50 million compounds from thousands of primary and confirmatory bioassays (these numbers seemingly grow everyday as more results are uploaded).[1] This activity data is regularly obtained in a two-step process: high-throughput screening (HTS), called a primary screen, of hundreds of thousands of compounds followed by a more detailed confirmatory screen for the compounds indicated as active in the HTS process. The HTS process is coarse, using microamounts of the compounds, and can regularly result in the identification of active compounds that often only number in a fraction of a percent of those tested. The confirmatory assay, conversely, would normally take the active compounds identified in the coarse screen and subject them to a more detailed analysis, both confirming their activity values and providing a quantitative value of activity, regularly reported as a concentration value at 50% inhibition or the IC_{50}.[2]

If one were to make a matrix that contained the compounds available in PubChem in columns and confirmatory bioassay results in rows for those compounds, that matrix would be overwhelmingly sparse. While such a matrix represents our vast holes in knowledge, it also provides an opportunity for the use of virtual screens in an attempt to mine known data and make predictions in the sparse areas of the matrix based on models.

Factor XI (FXI) is a key component of the intrinsic coagulation pathway, and consists of two polypeptide chains of 607 amino acids that are activated by thrombin (FXIIa) to form the FXa complex. The role of FXIa in hemostasis includes procoagulant action (the formation of fibrin) and antifibrinolytic action (the protection of fibrin).[3,4] Recent studies suggest that high levels of FXIa are associated with venous thrombins, which can lead to dangerous vascular occlusions.[4,5] There is considerable need to limit thrombosis and thromboembolism after major surgery or trauma to prevent occurrence of secondary complications. In the United States, Warfarin (4-hydroxy-3-(3-oxo-1-phenyl-butyl)chromen-2-one) is the anticoagulation drug of choice, which has rare but serious complications and interactions with other drugs. Warfarin is not a direct inhibitor of FXIa, but instead inhibits vitamin K-dependent synthesis of many of the clotting pathway factors.[6–8] Studies show that direct inhibition of FXIa can successfully decrease clotting responses in rabbit models.[9] These and other similar studies demonstrate the important role of FXIa in the three-dimensional (3-D) growth of arterial thrombi, which indicates that the inhibition of FXIa offers an antithrombotic target for prevention of atherothrombosis.[9,10] Therefore, identification of new chemicals or compounds that can inhibit the activity of FXIa with

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varied chemical and physical properties could have great pharmaceutical potential especially to regulate thrombosis and thromboembolism.

Virtual screening for the attempted identification of new lead compounds can be implemented in a variety of ways.[11] A typical approach includes performing a screening of a database of ligands for activity in docking studies and subsequently validating the predicted activity through experimentation. A classic example is the work of Shoichet and coworkers[12] who screened 2.35×10^5 compounds using docking to identify inhibitors of protein tyrosine phosphatase-1B. They identified 365 compounds in this way as predicted actives, of which about one-third were confirmed through experimentation. Other approaches include using a quantitative structure–activity relationship (QSAR), which is trained from a dataset and used to screen a larger dataset for potentially active compounds. For example, Zhang et al.[13] trained a QSAR model for antimalarial compounds and subsequently screened close to 5×10^5 compounds from the ChemBridge database. Of the predictive active compounds from the screen, 14% were experimentally verified as having antimalarial activity. Various modifications on virtual screening approaches can include the types of QSARs used, the molecular descriptors used to characterize the compounds or the screening metrics themselves. Ballester et al.[14] used a technique to identify inhibitors of arylamine *N*-acetyltransferases by screening molecular shapes from a database of about 7×10^5 compounds that were similar in shape to known inhibitors. This approach and subsequent experimental verification resulted in a hit rate of 40%. Combined methods can be used as well. Ji et al.[15] have screened the ZINC database using docking combined with a pharmocophore model to virtually identify inhibitors for marine alkaline protease. Of the top 10 compounds identified from this approach, three were verified experimentally as active.

Previously Visco and coworkers,[16] using a molecular descriptor called Signature, have performed a virtual screen to mine the PubChem compound database for inhibitors of Factor XIa (FXIa).[17] In this process, they first created a model to classify the results of a confirmatory bioassay (AID 846) from PubChem for 115 compounds against FXIa. Their model used the Signature molecular descriptor and a machine-learning process called a support vector machine (SVM) to successfully classify 89% of the compounds. With the inclusion of other refinement metrics fine-tuned by the primary screen (AID 798), they used this model to classify the (at the time) 12 million compounds from the PubChem compound database. They identified 296 compounds in this virtual screening whose activity values against FXIa they were most confident in. All 296 compounds identified were then successfully docked to the FXIa crystal structure, providing additional confidence in the inhibitors identified. The next step was to confirm the activity predictions experimentally, which is the primary objective of this letter.

Background on the Modeling Approach

Signature molecular descriptor

The Signature molecular descriptor is a powerful and robust tool used to encode the local environment near an atom in a molecule. Introduced about two decades ago in structural elucidation studies,[18] it has been used in a wide variety of areas since then, such as the prediction of protein–protein interactions,[19] process,[20] and molecular design,[21] and efficiently accounting for stereochemistry effects of bioactivity.[22]

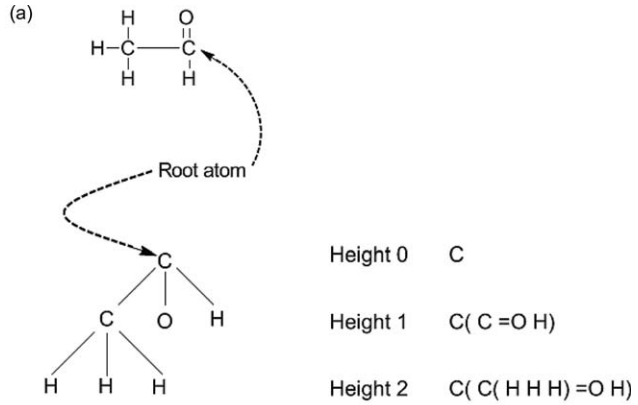

Figure 1. (a) The atomic Signatures at three heights for one of the carbon atoms in acetylaldehyde. (b) The molecular Signature for acetylaldehyde at height-1.

Briefly, an atomic Signature is a 2-D subgraph, starting with a root atom, and includes all atoms and bonds away from the root out to a predetermined layer (called a height). A molecular Signature of a molecule is then the sum of its atomic Signatures, with occurrence numbers as integer coefficients that identify the number of times a particular atomic Signature appears in a molecule.[16] In Figure 1, we provide the atomic signature for one atom in acetylaldehyde at various heights and provide the subsequent molecular signature for this substance at height 1. Two key features of the Signature molecular descriptor is that it has a tunable degeneracy (the molecular Signatures are practically nondegenerate by height-3), and it is easily invertible, meaning there is an efficient algorithm to generate structures from molecular Signatures.[23]

Support vector machine

A SVM is a machine-learning model that attempts to separate classes of observations plotted in the parameter space by planes (i.e., actually hyperplanes in the multidimensional space).[24] It is an optimization problem whose goal is to maximize the separation of the classes, with a penalty associated with a misclassification. A subset of the observations from both classes forms the support vectors, which define the margins between the classes. For a two-class problem (such as the one in the current work—active and inactive), the positive class is given a value of "+1" while the negative class is given a value of "−1". Once the model is trained on a set of data, it can be used to predict the classification of observations not part of the training. While any number greater than "0" can be considered part of the positive class and any number less than "0" part of the negative class, more

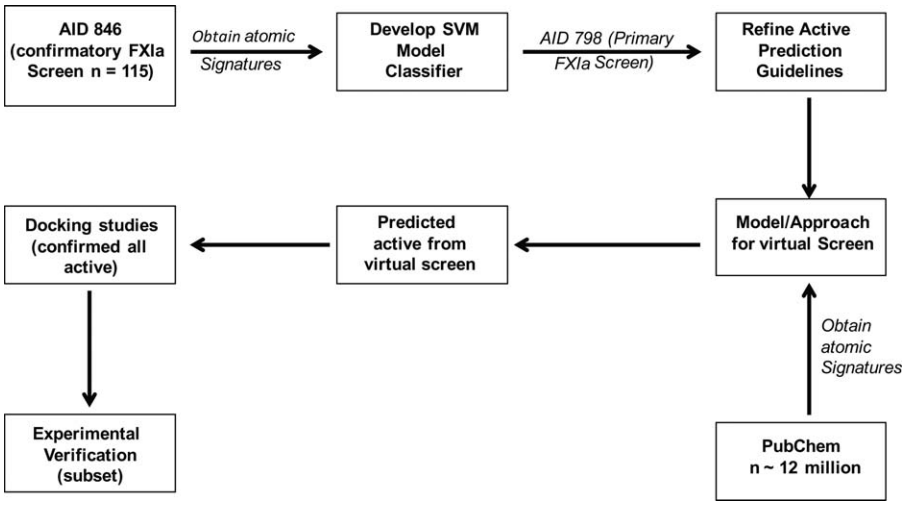

Figure 2. Computational primary screening and selection used to narrow target compounds for conformational assays.

confidence in class placement is derived from larger magnitude values (which can have a magnitude greater than 1).

Classification of AID 846

We used atomic Signatures of heights 0, 1, and 2 to deconstruct the 115 compounds of AID 846 and arrive at 865 unique height-1 atomic Signatures. After removing those atomic Signatures that only appear once (to limit the size of the problem), our atomic Signature database consisted of 411 atomic Signatures. We trained our SVM to classify the 115 compounds (47 active and 68 inactive) of AID 846 into the two classes using the 411 atomic Signatures. Without feature selection, the accuracy of the model obtained was 56%. However, once we used a *K*-means clustering with a 10-fold cross-validation, the accuracy of the model peaked at 89% (the optimal model obtained 22 clusters across 105 atomic Signatures).

Virtual screening of PubChem

Once the model was trained, it was used to explore the nearly 2×10^5 inactive compounds of the primary screen (AID 798) for FXIa as a test set. Through this test set, two things were learned: (1) the more the test set compounds had atomic Signatures that were used to develop the model, the more accurate the classification and (2) the larger the magnitude of the class prediction for the compound, the more accurate the classification. Indeed, when the overlap with the atomic Signatures from the model was total (100%) and the magnitude of the classification was larger than 2, all 1.442×10^3 compounds falling into this category were perfectly identified as inactive. Therefore, we used these general criteria for the virtual screening for the rest of PubChem to identify potentially active compounds against FXIa. From this, 296 compounds (of the nearly 1.2×10^7 available in PubChem at the time of that work) were identified whose atomic Signature overlap with the compounds in the model was at 100% and that had a model class prediction of at least +1. Figure 2 provides a view of the overall approach.

Methods and Materials

Inhibitor selection

Of the 296 compounds identified by our virtual screening, 21 were selected for further experimental evaluation. These compounds were chosen based on their structural similarity (or lack thereof) to the compounds in the confirmatory bioassay. The metric used was the Tanimoto Coefficient (TC; set-theoretic) and is a measure of structural similarity, ranging from similar (1) to dissimilar (0).[11] The TC values between each of the identified 296 compounds and those in the training set range from 0.36 to 1. Rather than an average similarity, we report the maximum TC for each compound tested relative to those compounds in AID 846. Additionally, two known FXIa inhibitors from AID 846 were also chosen as positive controls and evaluated experimentally to further confirm our experimental approach.

Materials

Human FXIa (activity: 193.20 activity U/mg, Enzyme Research Laboratories, South Bend, IN), Boc-Glu(OBzl)-Ala-Arg-AMC·HCl (Bachem Americas, Torrance, CA), Methylsulfoxide, 99.7, Extra Dry Over Molecular Sieve, AcroSeal^{TM} (DMSO; Cat. #: 34844-1000 VWR, Philadelphia, PA), Tris (Cat. #: 23004, Chem-Impex, Wood Dale, IL), sodium chloride (Cat. #: S5886, Sigma-Aldrich, St. Louis, MO), Tween 20 (P7949, Sigma-Aldrich). All inhibitor compounds are listed in Table 1, and were purchased from ChemBridge Corporation (San Diego, CA).

Flourometric enzyme inhibition assays

A fluorometric enzyme inhibition assay was used to test inhibitor activity and is based on AID 846 Pubchem Bioassay summary (FXIa 1536 HTS dose response confirmation—BioAssay summary).[25] The inhibitor reaction mechanism is shown by Figure 3. Briefly, the inhibitor stock (single compound from Table 1) was prepared at 1.0×10^{-4} mol/L in 20% DMSO, fluorescent peptide substrate (Boc-Glu(OBzl)-Ala-Arg-AMC·HCl; AMC = 7-amino-4-methylcoumarin fluorescent moiety) and enzyme (Human FXIa) were prepared at

Table 1. Inhibitor Activity Results

Compound	CID	Tanimoto Coefficient	IC_{50} (μM)
3-(2-Furyl)-1-(2-methoxybenzoyl)-5-(methylthio)-1H-1,2,4-triazole	*977140*		*0.36*[a]
N-[4-(1H-1,2,3-benzotriazol-1-ylcarbonyl)phenyl]butanamide	*220551*		*1.77*[a]
N-[4-(1H-1,2,3-benzotriazol-1-ylcarbonyl)phenyl]pentanamide	2211745	0.9756	1.7[a]
1-(4-fluorobenzoyl)-3-(4-methoxyphenyl)-5-(methylthio)-1H-1,2,4-triazole	976343	0.8478	21.13[a]
2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]-*N*-(4-phenyl-1,3-thiazol-2-yl)acetamide	1144816	0.4559	24.34[a]
1-(4-nitrobenzoyl)-1H-1,2,3-benzotriazole	710799	0.6944	29.89[a]
1-(3-nitrobenzoyl)-1H-1,2,3-benzotriazole	710710	0.6757	35.31[a]
1-(3,4-dimethoxybenzoyl)-2-(methylthio)-1H-benzimidazole	977731	0.8043	36.16[a]
3-(4-methoxyphenyl)-5-(methylthio)-1-(3-nitrobenzoyl)-1H-1,2,4-triazole	1288249	0.7692	40.40[a]
N-(3,4-dimethoxyphenyl)-2-(3,4-dimethyl-*N*-methylsulfonylanilino)acetamide	1361217	0.5714	> 50
2-oxo-4-propyl-2H-chromen-7-yl 2-furoate	807248	0.7255	> 50
2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]-*N*-(4-nitrophenyl)acetamide	2904743	0.4844	> 50
N-(3,4-dimethoxyphenyl)-1-benzothiophene-3-carboxamide	1243998	0.4808	> 50
3-(2-furyl)-5-(methylthio)-1-(3-nitrobenzoyl)-1H-1,2,4-triazole	976495	0.8478	> 50
N-(1,3-benzodioxol-5-yl)-2-(4-fluoro-*N*-methylsulfonylanilino)acetamide	1148866	0.4412	> 50
1-[4-(1-azepanylsulfonyl)benzoyl]-2-(methylthio)-1H-benzimidazole	1542203	0.4915	> 50
2-[2,3-dihydro-1,4-benzodioxin-6-yl(methylsulfonyl)amino]-*N*-(3-methylphenyl)acetamide	2194720	0.5385	> 50
2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]-*N*-(4-phenyl-1,3-thiazol-2-yl)acetamide	2275606	0.4559	> 50
4-(4-methoxyphenyl)-2-oxo-2H-chromen-7-yl 2-furoate	1334087	0.6304	> 50
2-{[(4-methoxyphenyl)sulfonyl]oxy}c-1H-isoindole-1,3(2H)-dione	2196958	0.7714	> 50
4-(2-chloro-4-methylbenzoyl)-4H-1,2,4-triazol-3-amine	973359	0.7949	> 50
2-[1,3-benzodioxol-5-yl(methylsulfonyl)amino]-*N*-[4-chloro-3-(trifluoromethyl)phenyl]acetamide	2231645	0.3947	> 50
N-(3-acetylphenyl)-2-[1,3-benzodioxol-5-yl(methylsulfonyl)amino]acetamide	2235746	0.4714	> 50

[a]Indicates active compound; compounds in italics are known FXIa inhibitors (positive controls); all associated chemical structures are provided in the Supporting Information.

6×10^{-5} mol/L and 9.2 μg/mL (193.20 activity U/mg) in assay buffer (50×10^{-3} mol/L Tris, pH = 7.4, 150×10^{-3} mol/L sodium chloride, 0.02% Tween 20). A one in five serial dilution of inhibitor was created spanning 1×10^{-4} to 3.2×10^{-8} mol/L. In a black 96-well plate, 0.1 mL of the serially diluted inhibitor solution was added to prepare the inhibitor working solution set. The control solutions were prepared using 0.1 mL of 20% DMSO-water solution instead of the inhibitor solution, while the blank control group contained 0.15 mL 20% DMSO-water solution. All samples and controls were tested in triplicate. Finally 5×10^{-2} mL of the fluorescent peptide substrate solution was transferred to

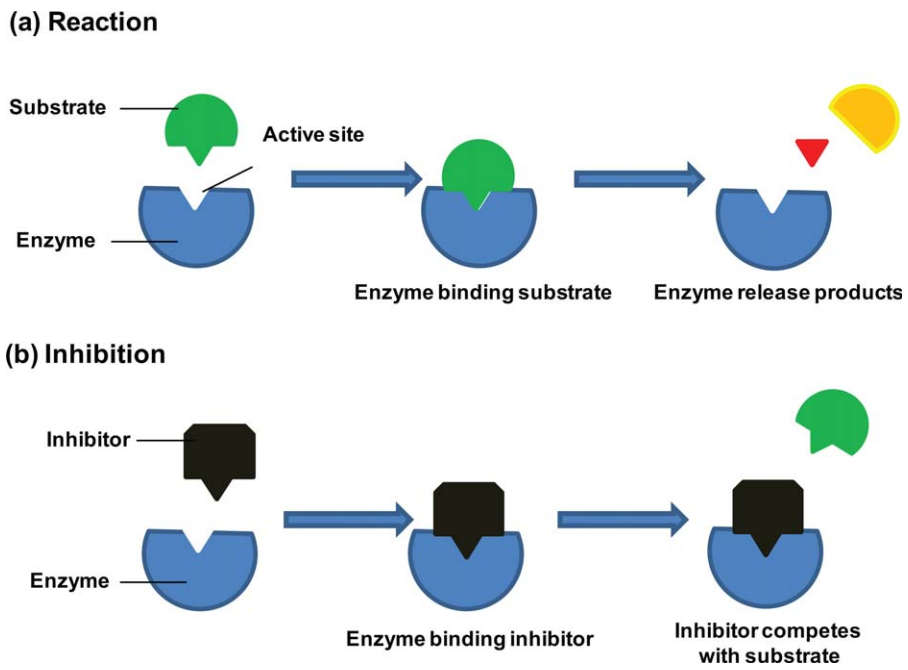

Figure 3. Human FXIa inhibitor assay reaction mechanism, (a) enzyme (FXIa) reacts with fluorogenic tripeptide substrate (Boc-Glu(OBzl)-Ala-Arg-AMC) to release the fluorescent AMC molecule for spectrofluorometer detection; (b) competitive inhibition of FXIa enzyme by binding to the same active site.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

all wells. The FXIa enzyme working solution was prepared by 100-fold dilution of the enzyme stock solution and 5×10^{-2} mL of this solution was added to all wells except the blanks. The well plates were protected from ambient light, placed on an orbital shaker and the reaction proceeded for 2 h at room temperature. Next, the fluorescence value (excitation 355 nm, emission 460 nm) were determined with a microplate reader (Tecan M200, San Jose, CA). The percent activity was calculated for each dilution of each compound from the signal in fluorescence units (FU) and the mean FU of the plate controls and the mean FU of the plate blanks using the following equation:

$$\text{Percent activity} = 100\% \times ((\text{signal} - \text{blank mean})/(\text{control mean} - \text{blank mean}))$$

Linear interpolation of the dilution curve is then used to determine the IC_{50} value for any compounds with a percent activity less than 50%, Activity outcomes were reported as follows: $IC_{50} < 50 \times 10^{-6}$ mol/L in all three IC_{50} determinations = active, $IC_{50} < 50 \times 10^{-6}$ mol/L in only 1 or 2 out of 3 determinations = inconclusive, $IC_{50} > 50 \times 10^{-6}$ mol/L, percent inhibition 30–50% at 50×10^{-6} mol/L = inconclusive, $IC_{50} > 50 \times 10^{-6}$ mol/L, percent inhibition <30% at 50×10^{-6} mol/L = inactive.[25] Data were exported and analyzed using Microsoft Excel.

Results

Optimization of the published FXIa assay[25] was required for testing the compounds listed in Table 1. Specifically we found that enzyme activity level (activity U/mg) was extremely important in determining IC_{50} levels, and that enzyme activity level shows batch-to-batch variation from the commercial supplier. Previous publications do not provide this information. Instead of using 9.2 μg/mL (published values), we used 9.2×10^{-2} μg/mL (193.20 activity U/mg) in all of our studies. The mean FXIa inhibition IC_{50} values obtained for the 23 compounds (21 new + 2 from AID 846) are shown in Table 1. Note that the first two compounds are from the active set in AID 846 and they are, as expected, active in our study as well. Of the 21 tested, seven were identified as active, yielding a 33% hit rate. The average standard deviation across all activity data was below 5%.

Discussion and Conclusions

The advantage of virtual screening is the ability to reveal new compounds that are more potent and/or structurally different than known molecules. In this study, a more active FXIa inhibitor than what is currently known was not revealed; however, several new dissimilar active compounds were confirmed. Structurally dissimilar compounds (relative to known inhibitors) provide additional pathways for new lead compound development as discussed in the introduction.

As previously mentioned, the TC provides a metric used to indicate structural similarity. One of the compounds identified in our virtual screen (CID 2211745) is structurally very similar to a known inhibitor of FXIa, CID 220551 (TC = 0.9756). Thus, it is not a surprise to find that this compound (CID 2211745) is also active against FXIa. Conversely, our model and virtual screen has identified a compound that has a much lower structural similarity to the compounds in AID 846, namely CID 1144816 (TC = 0.4559). In total, a 33% hit rate was realized in this work using the machine-learning classifier with the Signature molecular descriptor for FXIa on a virtual screen of PubChem. This is an order of magnitude more efficient than typical HTS, whose hit rates typically range between 0.1 and 5%,[26] and provides promise for using this virtual screening technique against many additional targets. Indeed, the experimental high-throughput primary screen previously performed for FXIa (AID 798) resulted in a hit rate of less than 0.025%,[25] clearly showing the benefit of the virtual screening approach at identifying active inhibitors for FXIa.

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