Design, Virtual Screening, and Synthesis of Antagonists of α_{IIb}β_{3} as Antiplatelet Agents

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Supporting Information

ABSTRACT: This article describes design, virtual screening, synthesis, and biological tests of novel α_{IIb}β_{3} antagonists, which inhibit platelet aggregation. Two types of α_{IIb}β_{3} antagonists were developed: those binding either closed or open form of the protein. At the first step, available experimental data were used to build QSAR models and ligand- and structure-based pharmacophore models and to select the most appropriate tool for ligand-to-protein docking. Virtual screening of publicly available databases (BioinfoDB, ZINC, Enamine data sets) with developed models resulted in no hits. Therefore, small focused libraries for two types of ligands were prepared on the basis of pharmacophore models. Their screening resulted in four potential ligands for open form of α_{IIb}β_{3} and four ligands for its closed form followed by their synthesis and in vitro tests. Experimental measurements of affinity for α_{IIb}β_{3} and ability to inhibit ADP-induced platelet aggregation (IC_{50}) showed that two designed ligands for the open form 4c and 4d (IC_{50} = 6.2 nM and 25 nM, respectively) and one for the closed form 12b (IC_{50} = 11 nM) were more potent than commercial antithrombotic Tirofiban (IC_{50} = 32 nM).

INTRODUCTION

Thrombus formation is the most important pathological mechanism underlying atherothrombotic diseases such as acute coronary syndromes and ischemic stroke/transient ischemic attack, which are responsible for elevated mortality worldwide1,2 and which are a platelet-mediated phenomenon. To start to form clots, platelets should be turned from the rested state to the activated one.3 Rupture of atherosclerotic plaques is supposed to be the main cause of arterial thrombus formation.4,5 This exposes such platelet activating proteins as tissue factor, von Willebrand factor, collagen, etc. Activated platelets are able to excrete other agonists of platelet activation such as adenosine diphosphate and thromboxane A2, which promote activation of adjacent platelets.6 Activated platelets change their shape and expose fibrinogen receptors, integrin α_{IIb}β_{3}, which change their conformation from bent conformation to extended conformation with closed headpiece (Figure 1). Then β-subunit moves away from α-subunit and the receptor goes into high-affinity state with open headpiece in which it binds fibrinogen and von Willebrand factor, resulting in clot formation and clot adherence, respectively.7 Thus, inhibition of α_{IIb}β_{3} can prevent clot formation regardless of the platelet activation pathway.8–10

Most antagonists of α_{IIb}β_{3} represent peptidomimetics, which mimic RGD or KGD sequence of fibrinogen and bind to the open form of the integrin (Figure 1, Ligand B). For a long time researchers focused their efforts on design of novel RGD-
peptidomimetic that resulted in two marketed drugs Tirofiban\(^1\) and Eptifibatide.\(^1\) The third marketed drug is Abciximab, a monoclonal antibody specific for an epitope on \(\beta_3\) subunit.\(^1\) The existed drugs have proven their efficiency in reducing a risk of peri-procedural myocardial infarction and in urgent target vessel revascularization during catheterization.\(^1\)\(^4\) However, these compounds have some drawbacks like inducing thrombocytopenia,\(^1\)\(^5\)\(^6\) which is supposed to be an immunological response of an organism on the conformational changes in integrin \(\alpha_{\text{III}}\beta_3\) upon binding with RGD-peptidomimetics.\(^7\)\(^8\)

Recently, a novel antagonist of \(\alpha_{\text{III}}\beta_3\), RUC-1, has been discovered during experimental screening of ~33 000 small compounds.\(^9\) According to mutagenesis studies, RUC-1 binds only to the \(\alpha_{\text{IIb}}\) subunit of the integrin. As it is shown in gel filtration and dynamic light scattering experiments, it does not induce transformations leading to open headpiece form (Figure 1, Ligand A). Later on, this was confirmed by X-ray study of the complex of RUC-1 and \(\alpha_{\text{III}}\beta_3\).\(^10\) Notice that RUC-1 has a relatively weak inhibition potency of ADP-induced aggregation tested on human platelet rich plasma (IC\(_{50}\) = 13 \(\mu\)M).\(^10\) In order to explore the RUC-1 binding pocket and to obtain additional information concerning binding mechanism and induction of conformational changes in the receptor, a series of derivatives of RUC-1 have been synthesized.\(^11\) One of them, named RUC-2, was found some 100 times more potent in inhibiting ADP-induced platelet aggregation than RUC-1 (IC\(_{50}\) = 96 nM).\(^12\) At the same time RUC-2 does not induce any conformational changes in the \(\alpha_{\text{III}}\beta_3\) headpiece,\(^12\) which may reduce adverse effects. Recently more potent ligands of the integrin’s closed form RUC-3 (IC\(_{50}\) = 45 nM) and RUC-4 (IC\(_{50}\) = 33 nM) have been reported.\(^13\) According to molecular docking and molecular dynamics simulations they interact with the same residues as RUC-2.

Protein–ligand binding patterns in \(\alpha_{\text{III}}\beta_3\) open and closed forms differ. Thus, Tirofiban binds with Asp224 residue of the \(\alpha_{\text{IIb}}\) subunit and with Mg\(^{2+}\) ion of metal ion-dependent adhesion site at the \(\beta_3\) subunit in the open form of integrin (Figure 2). However, RUC-2 binds to Asp224 residue of the \(\alpha_{\text{IIb}}\) subunit and to Glu220 residue of the \(\beta_3\) subunit, and thus, it displaces Mg\(^{2+}\) ion. These differences are key factors determining ligands effects on the conformational state of the receptor.

In this article, we report design of novel antagonists of \(\alpha_{\text{III}}\beta_3\) which bind to either open or to closed forms of the receptor. To achieve our goal we applied various ligand- and structure-based modeling techniques to screen either commercial databases or generated in this work virtual combinatorial libraries. Since antagonists of the open form have been investigated for a long time, a lot of data have been collected from in-house studies and from literature sources. Thus, for the design of the ligands for open form of \(\alpha_{\text{III}}\beta_3\) we used both ligand-based and structure-based approaches. Only few ligands of closed form of \(\alpha_{\text{III}}\beta_3\) have been reported in the literature. Therefore, here only structure-based approaches were used to design new compounds.

## RESULTS

### 1. Design of Antagonists of the Open Form of \(\alpha_{\text{III}}\beta_3\)

Two data sets have been collected from literature sources\(^{13\text{-}29}\) and in-house studies:\(^{13\text{-}29}\) (1) the affinity data set comprising 338 compounds with reported affinity values for \(\alpha_{\text{III}}\beta_3\) and (2) the antiaggregation activity data set comprising 453 compounds tested under similar conditions. These data sets were used for the development of QSAR and ligand-based pharmacophore models. X-ray structures of complexes of different antagonists with \(\alpha_{\text{III}}\beta_3\) have been used for the development of structure-based pharmacophore models and molecular docking. Ensemble of QSAR and pharmacophore models and docking experiments formed a virtual screening pipeline, which has been further used for compounds selection and prioritization.

#### 1.1. QSAR Models

Three individual 2D QSAR models both for ligands’ affinity and antiaggregation activity have been built using Random Forest\(^30\) method in combination with three types of fragment descriptors: simplexes (SiRMS),\(^31\)\(^32\) ISIDA fragments (SMF),\(^33\) and fuzzy pH-dependent pharmacophoric triplets (FPT).\(^34\)\(^35\) Consensus QSAR models have been developed by averaging predictions of the corresponding individual models. Predictive performance of the models was estimated by 5-fold external cross-validation. Usage of applicability domain (AD) for consensus models did not significantly improve prediction performance, see Table 1.

Relatively large RMSE values of predicted activities could be explained by several reasons. The first one is related to some heterogeneity of experimental data collected from different sources: experimental methods of evaluation of biological responses were not always similar. Different experimental

<table>
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<th></th>
<th>(R^2)</th>
<th>RMSE</th>
<th>(\text{AD}^{\text{RMSE}_{\text{AD}}}))</th>
<th>AD Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>affinity for (\alpha_{\text{III}}\beta_3)</td>
<td>0.75</td>
<td>0.76</td>
<td>0.76</td>
<td>0.72</td>
</tr>
<tr>
<td>antiaggregation activity</td>
<td>0.52</td>
<td>0.77</td>
<td>0.54</td>
<td>0.74</td>
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\(^{a}\)Statistical parameters take into account applicability domain.

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Figure 2. Interaction patterns of Tirofiban and RUC-2 compounds with integrin \(\alpha_{\text{III}}\beta_3\) in its open (left) and closed (right) forms.
conditions may significantly affect activity values. For example, usage of different agonists and anticoagulants in antiaggregation experiments may change measured activity values by up to 1 order of magnitude.\textsuperscript{36} Therefore, such data were not included in the data set. However, we accepted data measured by different experimental methods in order to collect a sufficiently big data set of compounds studied under similar conditions. The second reason is related to high interindividual variability of $\alpha_{\text{IIb}}\beta_3$ population in platelets, which can fluctuate in the range 71 700–10 200 receptors per platelet.\textsuperscript{37} This can lead to significant difference in both dissociation constants of fibrinogen ($K_D = 70–255$ nM)\textsuperscript{38} and affinity values of antagonists of $\alpha_{\text{IIb}}\beta_3$; for example, clinically relevant concentrations of Tirofiban, which cause inhibition of fibrinogen binding range from 17% to 88%.\textsuperscript{39} Clearly the above reasons introduce some inevitable noise in the data, which affects predictive performance of obtained QSAR models.

1.2. Pharmacophore Models. Three structure-based pharmacophore models were obtained with LigandScout\textsuperscript{40} from three available X-ray structures of $\alpha_{\text{IIb}}\beta_3$ complexes with small molecule antagonists L-739,758, Tirofiban, Eptifibatide (PDB codes 2VC2, 2VDM, and 2VDN, respectively).\textsuperscript{41} The binding pockets in these complexes are very similar: all major amino acid residues interacting with ligands are almost at the same positions. The models performance has been assessed in virtual screening of a validation set combining the affinity data set. Three ligand-based pharmacophore models are given in Figure S3 in the Supporting Material.

Example of initial and manually tuned structure-based pharmacophore models for Eptifibatide complex with $\alpha_{\text{IIb}}\beta_3$ (2VDN). The following labels for pharmacophore features were used: red stars, centers of negative charge; blue stars, centers of positive charge; red arrows — H-bond acceptors; green arrows — H-bond donors; yellow spheres, hydrophobic parts. Exclusion volumes are not shown for clarity.

Example of a ligand-based pharmacophore model. The following labels for pharmacophore features were used: red star, center of negative charge; blue star, center of positive charge; red arrow or sphere, H-bond acceptor; green arrow or sphere, H-bond donor; yellow sphere, hydrophobic part. Exclusion volumes are not shown for clarity. Notice that all other ligand-based pharmacophore models are given in Figure S3 in the Supporting Material.
was not clear, the docking calculations were performed on the structure with and without these water molecules. Computational experiments reveal overall performance of MOE applied to 2VDM binding site including water molecules (see Figure 6 and Table S2 in Supporting Information). This setup was further used in the virtual screening.

1.4. Design and Screening of the Focused Virtual Library. Virtual screening of BioinfoDB containing about three million of commercially available compounds with pharmacophore and QSAR models resulted in no hits. This can be explained by a low number of compounds with positively and negatively charged groups in commercial libraries. Even simple 2D pharmacophore representation (Figure 5) returns from BioInfoDB only 210 compounds. Subsequent screening with 3D pharmacophore and QSAR models resulted in no hits. Therefore, the focused virtual compound library has been created using a fragment-based approach. The main requirements for new antagonists of $\alpha_{IIb}\beta_3$ were derived from the pharmacophore models, docking studies, and some experimental observations. They are (i) positively and negatively charged groups should be separated by at least 16 Å; (ii) lipophilic fragment should be attached to the acidic part of a molecule; and (iii) desirable that the above lipophilic fragment is linked to a H-bond acceptor able to bind the Arg214 residue of $\alpha_{IIb}\beta_3$. According to these rules various Arg- and Asp-mimetic fragments and different linker groups were proposed (Figure 7). Combinatorial virtual library was generated by in-house computer program. After discarding synthetically irrelevant structures, the remaining 6930 compounds were used for the screening.

At the first step of virtual screening, pharmacophore and QSAR models were applied in parallel followed by selection of common hits (Figure 8). Hits selected by QSAR models met specified threshold values: for affinity $pIC_{50} \geq 8.0$ and for antiaggregation activity $pIC_{50} \geq 7.0$. Common application of
pharmacophore and QSAR models resulted in 93 hits representing 310 individual stereoisomers. Further selection with docking lead to 83 compounds (164 stereoisomers). All these compounds successfully passed through ADME/Tox filters using water solubility and Ames mutagenicity in-house models and toxicity assessment with PASS program. Two synthetically feasible compounds represented by two enantiomers each were chosen for further synthesis and biological evaluation.

It is interesting to note that the docking experiments revealed a little difference between binding poses of different enantiomers of selected compounds (Figure 9). In the complex, basic nitrogen of tetrahydroisoquinoline group binds to the Asp224, and carboxylic group of ligands binds to Mg$^{2+}$, whereas sulfonamide group forms H-bond with Arg214 residue. These interaction patterns are very similar to those in the Tirofiban-$\alpha$IIb$\beta_3$ complex. Thus, it can be expected that different enantiomers of designed compounds would have similar affinity values.

1.5. Synthesis and Biological Evaluation. Pure enantiomers of synthesized compounds were synthesized and tested for their affinity for $\alpha$IIb$\beta_3$ receptors and antiaggregation activity. For the purpose of comparison, experiments were also performed on the reference commercial compound Tirofiban.

DCC/SuOH method has been used for the preparation of previously reported RGDF mimetics, derivatives of 1,2,3,4-tetrahydroisoquinoline-7-carboxylic acid. This method was used on two stages when the acid 1 or 2 was coupled with sodium salts of appropriate $\beta$-alaines. The compound 2 described in this article was synthesized from the acid 1 and $\beta$-alanine using TSTU as a coupling reagent (Scheme 1). Acidolytic elimination of Boc-protective groups from compounds 3 yielded the target RGDF mimetics 4a–d.

Experimental data (Table 2) demonstrate high affinity for $\alpha$IIb$\beta_3$ and antiaggregation activity of compounds 4a–d. Tirofiban was used as standard inhibitor.

2. Design of Antagonists of the Closed Form of $\alpha$IIb$\beta_3$.

In this section we describe design of the analogues of RUC-2 ligand displaying high affinity for closed form of $\alpha$IIb$\beta_3$. Since very few experimental data on ligands for closed form were available, only structure-based pharmacophore and docking methods were used.

2.1. Pharmacophore Models. An initial structure-based pharmacophore model (Figure 10) has been generated with LigandScout using the structure of the RUC-2-$\alpha$IIb$\beta_3$ complex (PDB code 3T3M). This model contains: (i) two positive centers separated by 15.8 Å, (ii) five H-bond donors associated with positive centers, two of which directed toward $\alpha$IIbAsp224 amino acid and three to $\beta_3$Glu220, (iii) three H-bond acceptors associated with carbonyl group of the ligand, which binds to...
charged part (preferably pyperazine residue) able to interact with the Asp224 residue; (ii) a heterocyclic moiety interacting with the Tyr190 residue; (iii) an acceptor group (preferably carbonyl) interacting with the Asp232 residue, and (iv) positively charged part (amino group) displacing Mg$^{2+}$ ion and, in such a way, providing with interactions with Glu220 residue of the $\beta_3$ subunit. Potentially, a molecule combining 6-amino-2-(piperazin-1-yl)-3H-quainazolin-4-one scaffold connected to amino-group, as it is shown on Figure 11, may fulfill these conditions. Notice that substituted quinazolinolines and quinaizolinones derivatives are known as platelet aggregation inhibitors and fibrinogren receptor antagonists.\textsuperscript{54}

Based on these considerations, 29 virtual compounds (41 stereoisomers) were designed varying a linker separating 6-amino-2-(piperazin-1-yl)-3H-quainazolin-4-one scaffold and amino-group (see Figure 11).

Designed compounds were screened against 3D pharmacophore models, followed by docking with FlexX and application of ADME/Tox filters described in section 1.4. This resulted in 20 hits, three of which (compounds 12a–c in Table 3) were selected for the synthesis and biological tests. Their best docking poses (Figure 12) reveal the binding pattern similar to

![Figure 10. Pharmacophore model derived from the RUC-2-$\alpha_{\text{IIb}}\beta_3$ complex. Description of labels is given in the caption for Figure 3.](image)

**Table 3. Experimental Values of $\alpha_{\text{IIb}}\beta_3$ Antagonists of the Closed Form of the Receptor**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Affinity for $\alpha_{\text{IIb}}\beta_3$, nM</th>
<th>Anti-aggregation activity, IC$_{50}$, nM</th>
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<tbody>
<tr>
<td>12a</td>
<td>5.0 ± 0.8</td>
<td>150 ± 25</td>
</tr>
<tr>
<td>12b</td>
<td>2.2 ± 0.3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>12c</td>
<td>3.8 ± 0.4</td>
<td>100 ± 15</td>
</tr>
<tr>
<td>Tirofiban</td>
<td>2.4 ± 0.4</td>
<td>32 ± 4</td>
</tr>
</tbody>
</table>

![Figure 11. Schematic representation of ligands for closed form of $\alpha_{\text{IIb}}\beta_3$ used for generation of virtual focused library.](image)
those observed in X-ray RUC-2-αIIbβ3 complex : ligands’ amino group interacts with β3Asn215 and piperazin group with αIIbAsp224 (Figure 11, right). However, among 10 best poses we also discovered those with opposite orientation: amino group interacts with αIIbAsp224 and piperazine group, with β3Asn215 (Figure 11, left). These observations show that RUC-2 analogues may have different binding modes in the integrin’s binding pocket.

2.5. Synthesis and Biological Evaluation. In this section we describe a synthesis of compounds 12a−c selected in virtual screening. The synthetic route to a set of 2-piperazin-1-yl-quinazolines 12a−c is summarized in Scheme 2. Quinazoline-2,4-dione (6) was obtained by heating the isatoic anhydride and urea in DMFA.65 As a result of nitration of the compound 6, there was obtained the 6-nitro-3H-quinazoline-2,4-dione (7). There was carried out the chlorination of compound 7 using POCl3 to obtain 6-nitro-2,4-dichloro-quinazoline, which was further hydrolyzed to form 6-nitro-2-chloro-3H-quinazolin-4-one (8). The piperazyl ring was then conveniently introduced to the 2-position of the intermediate 8 by the reaction of it with the 1-Boc-piperazine, which gave the compound 9. The reduction of the nitro group of compound 9 using H2/Pd(C) gave the amine 10 as a crucial substrate for the construction of the target molecules. Condensation of Boc-protective groups yielded the compounds 12a−c.

Results of in vitro biology testing are summarized in Table 3. As one may see, all synthesized compounds are characterized by high affinity for αIIbβ3 and antiaggregation activity values.

**DISCUSSION**

This study demonstrated that virtual screening of large commercial databases resulted in no (for open form ligands) or very few (for closed form ligands) hits. This is not surprising taking into account that studied peptidomimetics or RUC-2 analogues have very specific features (charged parts separated by a certain distance), which do not occur in most of commercial compounds. That is why the only solution was generation of focused libraries followed by their screening. The lack of potential αIIbβ3 binders in commercial databases is confirmed in recent publications by Negri et al.58 and by Wang et al.36 reporting compounds with relatively weak antiaggregation potency (inhibition of ADP induced platelet aggregation IC50 = 12−47 μM58 and IC50 = 20−90 μM36) resulted from structure-based virtual screening of these data sources.
Important feature of the given study is application of different chemoinformatics approaches: QSAR, 2D and 3D pharmacophores, and ligand-to-protein docking. Joint application of different modeling techniques provided with very high success rate: almost all designed compounds displayed high affinity for $\alpha_{\text{IIb}}\beta_{3}$ and antiaggregation activity, and some of them perform better than commercial drug Tirofiban. Notice that the closed form ligand 12b (antiaggregation activity $IC_{50} = 11$ nM) designed in this study outperforms recently reported RUC-3 ($IC_{50} = 45$ nM) and RUC-4 ($IC_{50} = 33$ nM) molecules.  

**CONCLUSION**

This work is devoted to design, virtual screening, synthesis, and 

*in vitro* tests of novel $\alpha_{\text{IIb}}\beta_{3}$ antagonists. Two types of ligands were designed: those binding to open or to closed form of the protein. Various theoretical approaches were applied: QSAR, structure- and ligand-based pharmacophore and docking. Consensus virtual screening involving all these techniques allowed us to select very few molecules for the synthesis and 

*in vitro* tests. Experimental validation demonstrated very high success rate of our approach: 2 out of 4 hits for open-form ligands and 2 out of 4 hits for closed-form ligands demonstrated higher affinity and antiaggregation activity than commercial antiplatelet rich Tirofiban.

**EXPERIMENTAL SECTION**

**Description of Data Sets of RGD-Peptidomimetics Bound to the Open Form of $\alpha_{\text{IIb}}\beta_{3}$**. Two data sets of RGD-peptidomimetics, which possess affinity for $\alpha_{\text{IIb}}\beta_{3}$ or antiaggregation activity, have been provided by A.V. Bogatsky Physical-Chemical Institute of National Academy of Sciences of Ukraine (PCI). All of those compounds have been synthesized and tested for affinity to $\alpha_{\text{IIb}}\beta_{3}$ and antiaggregation activity at the Medicinal Chemistry Department of A.V. Bogatsky Physical-Chemical Institute by earlier described methods. Antiaggregation activity of compounds was measured by Born’s method on human platelet rich plasma. Affinity for $\alpha_{\text{IIb}}\beta_{3}$ was measured as inhibition of fluorescein isothiocyanate-labeled fibrinogen binding to activated human platelets by tested compounds. These two data sets contained relatively small number of compounds (45 compounds with reported affinity values and 53 with reported antiaggregation activity), and they were significantly imbalanced as they contained mostly active compounds. Due to this fact these data sets have been extended by data taken from CHEMBL database (version 7), which is a publicly available collection of organic compounds with reported bioactivity data. The compounds from CHEMBL database have been selected taking into account similarity of the used bioassays to those ones which were used in PCI tests. This was the crucial step because activity values for the same compound obtained in different assays can differ more than order of magnitude. Thus, we expect that in such a way prepared data sets should be less heterogeneous and more reliable for modeling.

Curation of the two data sets has been performed by Chemaxon Standardizer tool: (i) mixtures and inorganics were removed, (ii) salts were cleaned or removed, (iii) normalization of specific chemotypes (aromaticity and nitro groups were checked) were performed, (iv) explicit hydrogen atoms were added, and (v) treatment of tautomeric forms were done. The resulted data sets contained achiral and chiral compounds (single stereoisomers and racemic mixtures). During data set curation single stereoisomers were excluded if corresponding racememic compounds were present in the data set. Duplicates in the data sets have been removed using Chemaxon Instant JChem. All values of affinity for $\alpha_{\text{IIb}}\beta_{3}$ and antiaggregation activity of compounds were converted to $pIC_{50}$ ($-\log IC_{50}$; $IC_{50}$ in mol/L units). The extended data sets have become more balanced with better distributed and wider range of activity values (see Figure S1 in Supporting Information). These data sets are available as Supporting Information.

**QSAR Modeling of Open Form $\alpha_{\text{IIb}}\beta_{3}$ Antagonists.** Three different approaches for representation of molecular structure on 2D level have been used: simplex representation and two types of ISIDA descriptors, substructure molecular fragments and fuzzy pH-dependent pharmacophore triplets. As these approaches are well described in the literature we did not provide their detailed description here.

Random Forest method (implemented in the CF software) was used for QSAR models development because this method has proved its applicability for solution of various QSAR tasks. Predictive performance of obtained models, expressed as determination coefficient ($R^2$) and root mean-squared error (RMSE) (see eqs 1 and 2), has been assessed by 5-fold external cross-validation procedure. To perform 5-fold external cross validation all compounds in the data set were sorted according to their $pIC_{50}$ values, and each fifth compound went to the separate bin; thus, five bins were obtained. Then the compounds of four out of the five bins have been combined together, and QSAR model has been developed, compounds from the remaining fifth bin (which is actually an external test set) have been predicted by this model. This procedure was repeated five times to use all bins as an external test set only once. Predictions of external sets were combined and cross-validation statistics were calculated according to the eqs 1 and 2. Consensus predictions of affinity values for $\alpha_{\text{IIb}}\beta_{3}$ and antiaggregation activity of novel compounds have been made by averaging predictions of corresponding individual QSAR models.

$$ R^2 = 1 - \frac{\sum_{i=1}^{n}(y_{\text{exp},i} - y_{\text{pred},i})^2}{\sum_{i=1}^{n}(y_{\text{exp},i} - y_{\text{training}})^2} $$

(1)

$$ \text{RMSE} = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n}(y_{\text{pred},i} - y_{\text{exp},i})^2} $$

(2)

where $n$ is the number of compounds in an external set; $y_{\text{exp},i}$ is the observed activity value of $i$th compound in an external set; $y_{\text{pred},i}$ is the predicted activity value of $i$th compounds in an external set; $y_{\text{training}}$ is the mean activity value for compounds of a training set.

To check whether compounds were inside or outside of AD of the consensus models we estimated root-mean-square deviation of all predictions made by individual QSAR models. If a calculated standard deviation value was less than or equal to 0.5, then this compound was inside AD; otherwise, it was outside AD.

**ADME/Tox Assessments of Novel Compounds.** Some ADME/Tox properties of screening compounds, such as mutagenicity and solubility were estimated using previously reported QSAR models. The classification 2D QSAR model based on the Ames mutagenicity data set was applied for prediction of mutagenicity of novel compounds. This model was developed using Random Forest method and based on the simplex representation of molecular structure. The aqueous solubility of screened compounds was predicted by two 2D QSAR models in order to make average consensus prediction. The former is based on ISIDA descriptors and developed using Multiple Linear Regression method, and the latter was developed using Random Forest method in combination with simplex descriptors. Possible adverse effects were assessed by PASS program, which predicts probability of many types of activity and toxic effects.

**Pharmacophore Model Development.** Structure-based and ligand-based approaches implemented in LigandScout have been used for developing of 3D feature-based pharmacophore models. For generation of structure-based pharmacophore models ligands in complexes were ionized, and their energy was minimized using MMFF94 force field afterward structure-based pharmacophore models were produced.

**Ligand-Based Models of RGD-Peptidomimetics, Preparation of the Validation Set, and Considered Statistical Parameters.** Compounds of the affinity data set with $pIC_{50} \geq 8$ have been chosen for generation of ligand-based pharmacophore models. The selected compounds have been charged with Filter tool of OpenEye at and at most 200 conformers within 10 kcal/mol energy gap have been
generated using Omega.

Conformers having distance less than 16 Å between positively and negatively charged atoms (these are two key features of $\alpha$-helix, $\beta$-antagonists according to structure-based pharmacophore models) were discarded using in-house Python script based on OpenEye OEChem library.

Compounds have been clustered based on their pharmacophoric representation and for each of seven clusters of compounds a shared ligand-based pharmacophore has been generated with the default LigandScoot settings.

The validation set was prepared from compounds of the affinity data set and decoys. Compounds from the affinity data set were split on 234 active ($pIC_{50} \geq 6$) and 104 inactive ($pIC_{50} < 6$) ones. The set of inactive compounds has been extended by decoys selected from CHEMBL data set taking into account compounds similarity to known $\alpha$-helix, $\beta$-antagonists. At the first step, compounds with reported values of antiagregation activity or affinity for $\alpha$-helix have been discarded from the whole CHEMBL data set. Then, compounds that had the number of H-bond donor and acceptors, molecular weight, and topological surface area, predicted logD values within the range of corresponding values for compounds of the affinity data set have been chosen. On the last step, only 1518 compounds, which had at least one positively and one negatively charged group, have remained as these are key features according to the structure-based pharmacophore. Thus, the whole validation set contained 234 active compounds and 1622 inactives and decoys. Because some of active compounds were used in modeling of ligand-based pharmacophores they have been excluded from the set of actives for validation of ligand-based pharmacophore models.

All possible stereoisomers were generated for each compound in the validation data set having unspecified stereocenters. Afterward at most 200 conformers within 10 kcal/mol energy window were produced using Omega. The compound of the validation set was predicted as active if at least one of its stereoisomers fit at least one pharmacophore model. Pharmacophore fit scoring function taking into account only chemical feature overlap was used for ranking of the screening results.

Statistical characteristics that were used for estimation of pharmacophore models performance are given:

$$\text{recall} = \frac{TP}{TP + FN}$$
$$\text{precision} = \frac{TP}{TP + FP}$$
$$\text{enrichment ratio} = \frac{TP + FP}{TP + FN + TN + FP}$$

**Ligand-to-Protein Docking.** Three programs were used to carry out the docking studies: PLANTS, FlexX, and MOE. PLANTS uses stochastic search algorithm and chemplp scoring function. For the purpose of contact identification of rotatable bonds, charges, and protonation states of atoms, SPORES (structure recognition and protonation tool) was used. Centre and radius of the binding pocket were determined using PyMOL. Tuning parameters such as number of ants and iteration scaling factor remained by default.

Protein preparation for docking with FlexX consists of several steps: (i) the definition of the binding site by selection of the residues flanking the binding pocket; (ii) the check of ionization and tautomeric states, position of polar hydrogen atoms, crystal water molecules, and metal coordination type; and (iii) the addition of hydrogen atoms to fill out the remaining open valences of the receptor. FlexX uses systematic search method and its own empirical scoring function.

Binding pockets were prepared in Molecular Operation Environment (MOE) in several steps: (i) protonation of atoms of the protein, (ii) optimization of the protein–ligand complex, and (iii) removal of redundant water molecules. Stochastic search method and London D0 scoring function, which is implemented in MOE, were used in our docking studies.

Quality of self-docking and cross-docking studies was evaluated using RMSD values—root mean squared distance between corresponding atoms in an initial conformer and a docked pose. RMSD values lower than or equal to 2 Å were considered as satisfactory.

**Docking of RGD-Peptidomimetics.** Binding pockets of three selected complexes (PDB codes 2VC2, 2VD, and 2VDN) of $\alpha$-helix headpiece in the open form with three different ligands (L-739,758, Tirofiban, and Epitifibatide) were very similar in geometry: the root-mean-squared-distances (RMSD) between corresponding heavy atoms of residues included in binding pockets of different complexes were 0.8 Å for 2VD/2VC2 and 0.2 Å for 2VDN and 0.9 Å for 2VD/2VDN. Self- and cross-docking studies have been performed in order to choose the most appropriate binding pocket and estimate importance of water molecules coordinated with Mg$^{2+}$.

Results for Tirofiban and L-739,758, which were more similar to the studied ligands than Epitifibatide indicated that the presence of those water molecules was preferable (in the presence of the water molecules there was a greater number of good poses with RMSD ≤ 2 Å). It was found that MOE gives better results (lower RMSD values for Tirofiban and L-739,758 (1.15–2.14 Å) then FlexX (1.85–3.55 Å) and PLANTS (2.07–8.87 Å) (for details see Table S2 in Supporting Information). To make a final decision, compounds from the affinity data set were docked into 2VDM binding site using all three programs. To produce ROC curves to estimate docking performance compound of the affinity data set has been split on active ($pIC_{50} \geq 6.5$) and inactive ($pIC_{50} < 6.5$) ones. MOE demonstrated better performance (AUC = 0.72) against this set of compounds than PLANTS (AUC = 0.59) and FlexX (AUC = 0.49) (Figure 6). Therefore, MOE software and 2VDM binding site were chosen to perform virtual screening.

**Virtual Screening of Publicly Available Data Sets and Cofocused Libraries.** All compounds before screening were standardized with Chemaxon Standardizer and charged with Filter tool of OpenEye. The virtual screening workflow was common for both studies. On the first step 2D pharmacophore model implemented as in-house Python script based on OpenEye OEChem library has been applied to reduce the number of compounds to the reasonable level (several hundred or thousand). For screening of focused libraries this step was omitted because they had been created taking into account information from 2D pharmacophore. Then, 3D pharmacophores, QSAR models (if available), and docking have been applied. On the last step some ADME/Tox properties and possible side effects have been estimated, and synthetically feasible compounds have been selected for synthesis and experimental evaluation.

**Chemistry.**$^1$H NMR spectra were recorded on Bruker Avance DRX 300 spectrometer with chemical shifts in ppm with the internal TMS as a standard. Electron ionization (EI) and fast-atom bombardment (FAB) mass spectra were recorded on a VG Analytical VG 70-70EQ instrument. FAB spectra were performed equipped with an argon primary atom beam, and an m-nitrobenzyl alcohol matrix was utilized. The purity was measured by HPLC conducted on an Shumadzu system (System Controller CBM-20A, two pumps LC-8A, and Photodiode Array detector SPD-M20A) using a Hypersil GOLD 3 μm (4.6 mm × 150 mm) or Hypersil GOLD AQ 3 μm (4.6 mm × 150 mm) column. For preparative HPLC Fraction Collector FRC-10A was used. The progress of reactions was monitored by TLC (silica gel 60 F254, Merck).

The acid 1 has been synthesized using a previously published method.$^{17}$ The optically active $\alpha$-sulfonamido-$\beta$-alanines were prepared from L- or D-asparagine using previously published methods.$^{18,19}$

The compound 1 (2.0 g, 0.0072 mol) was dissolved in anhydrous acetonitrile (30 mL). The solution was cooled to −5 °C, and triethylamine (1.0 mL, 0.0072 mol) and then TSTU (2.168 g, 0.0072 mol) were added. The mixture was stirred for 1 h at...
−5 °C, and then a solution of β-alanine (1.283 g, 0.0144 mol) and NaHCO₃ (1.21 g, 0.0144 mol) in water (20 mL) was added. The reaction mixture was mixed 3 h at room temperature. The solvent was evaporated in vacuo to dryness. Then water (20 mL) was added, and pH of the mixture was brought to 3. The product was extracted from water by chloroform (2 × 150 mL). The organic layer was washed by 1 N HCl (2 × 50 mL) and water (50 mL), then dried with Na₂SO₄ and filtered, and the solvent was evaporated in vacuo. The resulting oily residue was dissolved in ether (50 mL), and the precipitate was collected by filtration and dried. Yield 81%; mp = 122 °C. ¹H NMR spectral and FAB-MS characteristics of the same product 2 previously obtained.⁷

General Procedure for a Preparation of Compounds 3. The compound 2 (0.5 g, 0.0014 mol) was dissolved in anhydrous acetonitrile (30 mL). The solution was cooled to −5 °C, and triethylamine (0.2 mL, 0.0014 mol) and then PFTU (0.6 g, 0.0072 mol) were added. The mixture was stirred for 1 h at −5 °C. The solvent was evaporated in vacuo to dryness. The residue was dissolved in 100 mL of chloroform. The solution was washed with aqueous solution of 1 M HCl (40 mL), 5% aqueous solution of NaHCO₃ (40 mL), and water (40 mL). The organic layer was dried over Na₂SO₄ and filtered, and the solvent was evaporated in vacuo to dryness. The resulting oily residue was dissolved in acetonitrile (30 mL), and then solution of α-substituted-β-alanine (0.0028 mol) and NaHCO₃ (0.253 g, 0.0028 mol) in water (20 mL) was added. The reaction mixture was mixed 2 h at room temperature. The solvent was evaporated in vacuo to dryness. Then water (20 mL) was added, and pH of the mixture was brought to 3. The product was extracted from water by ethyl acetate (2 × 100 mL). The organic layer was washed by 1 N HCl (2 × 20 mL), water (20 mL), then dried with Na₂SO₄, filtered, and the solvent was evaporated in vacuo to dryness. The resulting oil was dissolved in acetonitrile and was separated by 100 μL portions by preparative HPLC using the conditions: column, Hypersil GOLD AQ 5 μm (20 mm × 150 mm); flow rate, 20 mL/min; mobile phase, water/acetonitrile; isocratic 60/40; detection, UV 254 nm. The product containing fractions was combined, and the solvent was evaporated in vacuo to dryness.

2-(S)-(n-Butylsulfonyl)-3-{3-(2-BOC-1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (3a). Prepared from acid 2 and 2-(S)-(n-butylsulfonyl)amino-β-alanine. Yield 43%. A colorless glass substance; ¹H NMR δ (500 MHz, d₅-DMSO) 0.85 (t, J = 7.3 Hz, 3 H), 1.30–1.37 (m, 2 H), 1.42 (c, 9 H), 1.56–1.68 (m, 2 H), 2.36 (t, J = 6.7 Hz, 2 H), 2.80 (t, J = 5.4 Hz, 2 H), 2.96 (t, J = 3.3 Hz, 2 H), 3.17 (m, 4 H), 3.55 (m, 3 H), 4.35 (m, 3 H), 7.29 (d, J = 4.9 Hz, 2 H), 3.82–3.88 (m, 1 H), 4.52 (s, 2 H), 7.20–7.23 (m, 2 H), 7.62–7.64 (m, 2 H), 8.00 (s, 1 H), 8.41 (t, J = 5.1 Hz, 1 H); MS (FAB) m/z: 555 [M + H]⁺.

2-(R)-(n-Butylsulfonyl)-3-{3-(2-BOC-1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (3b). Prepared from acid 2 and 2-(R)-(n-butylsulfonyl)amino-β-alanine. Yield 38%. A colorless oil substance. ¹H NMR spectral and FAB-MS characteristics of the same product 3a.

2-(S)-(Phenylsulfonyl)-3-{3-(2-BOC-1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (3c). Prepared from acid 2 and 2-(S)-(phenylsulfonyl)amino-β-alanine.⁴ Yield 36%. A colorless glass substance; ¹H NMR δ (500 MHz, d₅-DMSO) 1.42 (c, 9 H), 2.27 (dddx, J = 28.5 Hz, J = 14.4 Hz, J = 7.6 Hz, 2 H), 2.80 (t, J = 5.5 Hz, 2 H), 3.09–3.15 (m, 1 H), 3.29–3.34 (m, 1 H), 3.36–3.41 (m, 2 H), 3.55 (t, J = 5.0 Hz, 2 H), 3.90 (dd, J = 14.7 Hz, J = 7.1 Hz, 1 H), 4.53 (s, 2 H), 7.22 (d, J = 8.2 Hz, 1H), 7.53–7.63 (m, 6 H), 7.77 (d, J = 7.4 Hz, 2 H), 7.99 (t, J = 5.5 Hz, 1 H), 8.56 (t, J = 5.5 Hz, 1 H); MS (FAB) m/z: 575 [M + H]⁺.

2-(R)-(Phenylsulfonyl)-3-{3-(2-BOC-1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (3d). Prepared from acid 2 and 2-(R)-(phenylsulfonyl)amino-β-alanine. Yield 51%. A colorless glass substance. ¹H NMR spectral and FAB-MS characteristics of the same product 3c.

General Procedure for a Preparation of Compounds 4. The compounds 3 (1 mmol) were dissolved in anhydrous CH₂Cl₂ (50 mL), and the stream of dry HCl was passed through the solution for 30 min. The solvent was evaporated, and the solid residue was dried in vacuo (2 mmHg) for 2 h at 40 °C.

Chloride 2-(S)-(n-Butylsulfonyl)-3-{3-(1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (4a). Yield 98%. A hygroscopic solid; ¹H NMR δ (500 MHz, d₅-DMSO) 0.86 (t, J = 7.1 Hz, 3 H, C₅H(CH₂)₃), 1.59–1.69 (m, 2 H, CH₂(CH₂)₂), 2.37 (t, J = 6.9 Hz, 2 H, NHCH(CH₂)CO₂H), 2.97 (dt, J = 15.4 Hz, J = 8.1 Hz, 2 H, CH₂Bu), 3.04 (t, J = 5.2 Hz, 2 H, CH₂THIQ), 3.22–3.27 (m, 1 H, C₃H(CH₃-NHSO₂Bu)CO₂H), 3.35–3.44 (m, 5 H, C₃H₂(CH₃-NHSO₂Bu)CO₂H and C₃H₂THIQ and NHCH(CH₂)CO₂H), 3.99 (dd, J = 15.3 Hz, J = 6.4 Hz, 1 H, CH₃(CH₃-NHSO₂Bu)CO₂H), 4.28 (s, 2 H, C₃H₂THIQ), 7.29 (d, J = 8.0 Hz, 1 H, CH₂THIQ), 7.56 (d, J = 9.0 Hz, 1 H, NHSO₂Bu)₂, 7.70–7.72 (m, 2 H, C₃H₂ and C₃H₂THIQ), 8.12 (t, J = 5.1 Hz, 1 H, NHCH(CH₃-NHSO₂Bu)CO₂H), 8.48 (t, J = 5.1 Hz, 1 H, NHCH(CH₃)₂, 9.58 (s, 2 H, NH₂THIQ). HRMS (FAB) calculated for C₃₅H₅₄N₅O₇S 7681 [M + H]⁺; found, 7681.5659.

Chloride 2-(R)-(n-Butylsulfonyl)-3-{3-(1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (4b). Yield 95%. A hygroscopic solid; ¹H NMR spectral and HRMS (FAB) characteristics of the same product 4a.

Chloride 2-(S)-(Phenylsulfonyl)-3-{3-(1,2,3,4-tetrahydroisoquinolin-7-yl)-carbonyl}amino[propionyl]aminopropionic Acid (4c). Yield 98%. A hygroscopic solid; ¹H NMR spectral and HRMS (FAB) characteristics of the same product 4a.
at 70 °C. Reaction mixture was allowed to cool to room temperature, and the precipitate of crude product was collected by filtration. Recrystallization from mixture of benzene and ethanol (2:1) to give pure compound 9. Yield 82%; mp 214–216 °C; 1H NMR δ (500 MHz, d6-DMSO) 1.42 (s, 9 H), 3.43 (br s, 4 H), 3.72 (br s, 4 H), 7.30 (d, J = 8.5 Hz, 1 H), 8.27 (d, J = 7.4 Hz, 1 H), 8.59 (s, 1 H), 11.52 (br s, 1 H); MS (FAB) m/z 376 [M + H]+.

2-(4-Boc-piperazin-1-yl)-6-amino-3H-quinazolin-4-one (10). The compound 9 (0.5 g, 0.0013 mol) was dissolved in anhydrous acetonitrile (25 mL). The solution was cooled to −5 °C, and triethylamine (1.4 mL, 0.01 mol) was then HATU (0.5 g). After reaction, the mixture was stirred for 1 h at 50 °C. After the reaction, the mixture was stirred for 7 h at 50 °C. The residual amount of the activated ether (At-ether of starting Boc-acid) was destroyed by addition of NaHCO3 (40 mL). The organic layer was dried over Na2SO4 and the solvent was evaporated in vacuo to give the precipitate of crude product was collected by filtration. Recrystallization from mixture of benzene and ethanol (2:1) to give pure compound 9. Yield 94%; mp 241–245 °C; 1H NMR δ (500 MHz, d6-DMSO) 1.37 (s, 9 H), 1.42 (s, 9 H), 2.51–2.55 (m, 2 H), 3.22 (s, 2 H), 3.40 (br s, 4 H), 3.55 (br s, 4 H), 6.86 (s, 1 H), 7.24 (d, J = 6.9 Hz, 1 H), 7.70 (d, J = 6.7 Hz, 1 H), 8.29 (s, 1 H), 10.01 (s, 1 H); MS (FAB) m/z 346 [M + H]+.

General Procedure for a Preparation of Compounds 11. The 0.01 mol of Boc-acid was dissolved in anhydrous acetonitrile (25 mL). The solution was cooled to −5 °C, and triethylamine (1.4 mL, 0.01 mol) was then HATU (0.5 g). After reaction, the mixture was stirred for 1 h at 50 °C. The residual amount of the activated ether (At-ether of starting Boc-acid) was destroyed by addition of NaHCO3 (40 mL). The organic layer was dried over Na2SO4 and the solvent was evaporated in vacuo to dryness. The residue was dissolved in 100 mL of chloroform. The solution was washed with water (40 mL), aqueous solution of 1 M HCl (40 mL), and 5% aqueous solution of NaHCO3 (40 mL). The organic layer was dried over Na2SO4 and the solvent was evaporated in vacuo.

**In Vitro Biology.** Functional activity was determined by measuring the inhibition of ADP induced platelet aggregation in human platelet-rich plasma (PRP) by Born’s method.36 Mode of action for some compounds was subsequently revealed in vitro by measuring the ability of compounds to inhibit the binding of fluorescein isothiocyanate-labeled fibrinogen (FITC-Fg)35 to αIIbβ3 in a suspension of human washed platelets.36

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00865.

Additional figures and tables illustrating the data sets, pharmacophores and molecular docking results (PDF)

Synthesized compounds and corresponding data (CSV)

Ligands of open form of αIIbβ3 with known affinity for αIIbβ3 which were used for QSAR, pharmacophore modeling and molecular docking studies (CSV)

Ligands of open form of αIIbβ3 with known anti-aggregation activity which were used for QSAR modeling (CSV)

Hits retrieved in virtual screening for the ligands of the open form of αIIbβ3 (CSV)

Hits retrieved in virtual screening for the ligands of the closed form of αIIbβ3 (CSV)

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**Notes**

The authors declare no competing financial interest.

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