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ORIGINAL PAPER

Rational design of antithrombotic peptides to target the von Willebrand Factor (vWf) - GPIb integrin interaction

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Abstract Conventional antithrombotic drug discovery requires testing of large numbers of drug candidates. We used computer-aided macromolecular interaction assessment (MIAX) to select antithrombotic molecules that mimic and therefore block platelet GPIb's binding to von Willebrand factor (vWf), an early step in thrombus formation. We screened a random array of 15-mer D-amino acid peptides for binding vWf. Structures of 4 candidate peptides were inferred by comparison to sequences in protein databases, conversion from the L to D conformations and molecular dynamics (MD) determinations of those most energetically stable. By MIAX, we deduced the amino acids and intermolecular hydrogen bonds contributing to the GPIb-vWf interaction interface. We docked the peptides onto vWf in silico to localize their binding sites and consequent potential for preventing GPIb-vWf binding. In vitro inhibition of ristocetin-initiated platelet agglutination confirmed peptide function and suitability for antithrombotic development, thereby validating this novel approach to drug discovery.

Keywords Antithrombotic design · Computer-aided drug design · GPIb-vWf interaction · Molecular soft docking · Peptide array · Peptidomimetic · Rational drug design

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Introduction

Platelets' ability to localize to a site of tissue or vascular injury and to participate in thrombus formation involves a number of concurrent and sequential processes that culminate in the arrest of bleeding. Conversely, inappropriate thrombus formation in the vasculature is the basis of heart attacks and strokes. At the molecular level, the critical events in thrombus formation, the adhesion and aggregation of platelets, are mediated by platelet integrins, GPIb, which upon conformational activation, binds to von Willebrand factor (vWf) [1, 2] and GPIIbIIIa, which binds fibrinogen [3]. The classical antithrombotics such as acetylsalicylate or ticlopidine target platelet function [4]. However, the newer antithrombotics are designed to disrupt the GPIIbIIIa - fibrinogen interaction by competition (*e.g.*, eptifibatide) or blocking (Abciximab) [4, 5]. Small molecules that mimic the interaction sites of such receptor-ligand pairs, such as a variety of RGD sequences, or structures based on them also can specifically block these adhesive molecules on the platelet surface [5]. A large number of such inhibitors of the GPIIbIIIa-fibrinogen interaction have been documented [5]. Although the GPIb-vWf interaction has been less of a focus of drug development, some inhibitors of that interaction exist as well [6].

We wished to design a peptidomimetic that would exert GPIb-like receptor function and antithrombotic function by inhibiting the GPIb-vWf protein-protein interaction. To do this, first, using bioinformatics, we defined the atoms involved in the interaction interface between GPIb and vWf. Next, using classical laboratory methods, we selected peptides from a random D-peptide library by their ability to bind to purified vWf. Returning to the computer, we then confirmed the site of the peptides' interactions with vWf and selected four peptides that best occupied a region within the GPIb-vWf recognition interface. This report describes the analysis of these random D-peptides for structural characteristics that would allow them to target the interface between vWf and GPIb and included exhaustive computer modeling of affinity to define the candidate peptide structures. We now describe the computational evaluation of the protein-peptide complexes that, through computational methodologies, have the highest probabilities of being formed, thereby confirming the laboratory observations. We investigated the characteristics of the complexes and their stability in silico, and then synthesized some of the peptides to provide laboratory confirmation of inhibitory function.

Commercial software for computational methodologies that allow these types of evaluations is not available; therefore, we carried out the study using a suite of programs developed in our laboratories. Central to this collection of computational procedures is the MIAX paradigm (macromolecular interaction assessment computer system) [7–12] which enables the prediction of the most probable configuration of protein-protein, protein-peptide, and other bio-macromolecular complexes in solution.

It combines in a rational way a series of computational methodologies, the goal being the prediction of the most native-like protein complex that may be formed when two isolated (unbound) protein monomers interact in a liquid environment. The overall strategy consists of first inferring putative pre-complex structures by identification of binding sites or epitopes on the proteins' surfaces and a simultaneous rigid-body docking process using geometric instances alone. Pre-complex configurations are defined here as all those decoys of which the interfaces comply substantially with the inferred binding sites and whose free energy values are the lowest. Retaining all those pre-complex configurations with low energies leads to a reasonable number of decoys for which a flexible treatment is amenable. MIAX is endowed of novel algorithmi for automatically inferring binding sites in proteins given their 3-D structure. The procedure combines an unsupervised learning algorithm based on the self-organizing map or Kohonen network with a 2-D Fourier spectral analysis. To model interactions, the potential function proposed here plays a central role in the system and is constituted by empirical terms expressing well-characterized factors influencing bio-macromolecular interaction processes, essentially electrostatic, van der Waals, and hydrophobic ones. Each of these procedures has been validated by comparing results with observed instances. Finally, the more demanding process of flexible docking is performed in MIAX embedding the potential function in a simulated annealing optimization procedure. Whereas search of the entire configuration hyperspace is a major factor precluding hitherto described systems from efficiently modeling macromolecular interaction modes and complex structures, the paradigm presented in MIAX involving the treatment of the information available from the 3-D structure of the interacting monomers combined with conveniently selected computational techniques assist to exclude the search of regions of low probability in configuration space and indeed leads to a highly efficient system oriented to solve fundamental biological problems.

We used MIAX to analyze and predict biomolecular structures, assess and infer inter-macromolecular interactions, and assist the rational process of drug design. The latter process is epitomized by the ability of the system to infer and thus assist in the design of complementary peptides to protein active sites particularly those located within the interface of protein-protein interactions. MIAX is oriented to the holistic analysis of the structure and function of proteins and other bio-molecules translated in their interaction patterns with other protein subunits or other biomacromolecules as well as with small organic compounds. MIAX can be used to perform the analysis when independently crystallized proteins known to interact are introduced to the system. Similarly, a complex structure can be introduced to determine the interaction trajectory and typify the interaction interface as well as its composition in terms of the amino acids involved in the interaction.

Complexes output by MIAX are tested for stability using molecular dynamics (MD) methods. The results show that three out of the selected four peptides bind to regions in the interface of interaction between vWf and GPIb. Stability of the vWf-binding peptides is high since MD simulations performed for several pico-seconds hardly distort the complex output by MIAX. Furthermore a hydrophobic complementarity as well as the network of hydrogen bonds can clearly be mapped among the interacting units in the three cases of high affinity peptides. These analyses and several others discussed in the methodology section unveil the most important forces at the atomic level that contribute to the binding of the peptides to vWf, and reinforce the postulated complex configurations.

Materials and methods

We applied MIAX to the complex of GPIb integrin and its ligand, vWf, found in the Protein Data Bank (PDB:1SQ0) and analyzed the GPIb-vWf system to determine the characteristics of the interaction interface of the resulting complex. We devised a methodology that consists of six steps performed recursively for each of the peptides to evaluate its interaction with vWf.

Characterization of the interaction interface of the GPIb-vWf protein complex

Characterization of the interaction interface for the GPIb-vWf complex is performed by computing the decrement in surface area of the subunits at complex formation. SASA is computed with a water molecule radius of 1.4 A. The amino acids' differences in SASA identify them as those involved or not in the interaction interface. Computing distances between atoms belonging to different units in the interaction allows inference of particular interactions between the units such as hydrogen bonds, electrostatic interactions or hydrophobic interactions, which can be compared with reported interactions or with those in the entries of interaction databases.

Physicochemical characteristics of the interaction interfaces

Physicochemical characteristics of the interacting subunits (interacting proteins and peptides) are computed by means of the SOM-MIAX module in MIAX [11]. The main physicochemical characteristic computed for GPIb-vWf is the relative hydrophobocity of regions on the proteins' surfaces. The calculation uses the molecular hydrophobic potential introduced by Brasseur [13], and a learning algorithm that incorporates the self-organized maps of Kohonen [14]. Image processing is applied to define the limits of the hydrophobic patches on the surfaces of the interacting units.

Generation of inhibitory peptide sequences

Random peptide arrays of 1120 peptides made of D-amino acids were synthesized on a cellulose membrane using an AutoSpot ASP 222 peptide synthesizer (ABiMED, Langenfeld, Germany). Resulting replicate libraries of 15-mer sequences were probed for vWf binding function by exposing the membranes to purified vWf (a gift of Dr. F. A. Ofosu, McMaster University, Hamilton ON, Canada) after blocking with skim milk to prevent non-specific binding of horseradish peroxidase labeled goat anti-human-vWf IgG. (Cedarlane, Canada) used to identify positive spots. The chemiluminescent substrate from the Amersham Pharmacia ECL kit detected positive spots that were recorded on photographic film. Negative controls consisted of probing the membranes with the antibodies without prior exposure of the membrane to purified vWf.

Modeling the 3D structures of the designed peptides

The three dimensional structures (3D) of peptides can be determined by ab initio calculations such as the GAX system [15]. This is a robust methodology to build 3D structures of the peptides designed to bind vWf. The Brookhaven PDB [16] was scanned for segments of high similarity to the sequences of the selected peptides. A FASTA [17] search identified highly similar sequences and their structures were used as the initial conformations for the peptides. The 3D structures underwent a change from the L conformation to the D conformation and a series of minimizations and molecular dynamics simulations produced the most energetically stable conformations for the peptides in solution. These were performed using the force fields in AMBER-6 [18].

Docking of the peptides to a receptor using MIAX

With the 3D structures of the interacting molecular entities, the docking module of MIAX [7] computed the complexes they may form when they interact. MIAX is endowed with three types of modules for docking macromolecules: a rigid body docking module to discover interaction pathways when the structure of the complex is known a priori; a "soft docking" module, that docks two units of which the structures are known only in the isolated state. This being the present case, this module was applied first to dock the peptides to vWf. The third module in MIAX is characterized by the flexible docking of units, in which there is a rigorous analysis of the conformation of the side chains of interface amino acids. MIAX performs the docking taking into account the geometry of the molecules as well as the interaction energy of the system.

Geometric characteristics of the interacting subunits are considered by a discretization process of the molecular bodies and performing a grid point complementarity analysis of the subunits and their fit into 3D space [11]. The interaction energies are computed by the following expression:

$$\Delta G^{AB(s)} = E_{hy} + E_{elec} + E_{hb} + E_{tor} + E_{desol}, \quad (1)$$

where $\Delta G^{AB(s)}$ is the change in free energy at complex formation in solution, and the terms on the right stand for the hydrophobic energy (E_{hy}), electrostatic interaction (E_{elec}), hydrogen bonding (E_{hb}), torsional energy (E_{tor}) and the energy of desolvation (E_{desolv}). Each of these terms is described in detail elsewhere [11].

Molecular dynamics simulation of the complexes to compute complex stability

The stability of the complexes obtained by the MIAX docking process is tested by means of molecular dynamic simulations using the AMBER-6 force field [17]. The simulation is performed in vacuum and for 50 ps for each of the complexes. The second objective of this simulation is to detect any major change in the conformation of the subunits, *eg* changes in the interaction interface that may lead to improved accommodation of the peptide on vWf.

Characterization of peptide-vWf interaction interfaces and validation of the selected peptides

Characterization of the interaction interfaces of the candidate conformations (decoys) for the peptide-vWf complex output by MIAX followed by the molecular dynamics experiment was done as for computing the interaction interface of GPIb-vWf complex. The decrement of SASA of atoms constituting the peptides and vWf led to the map of the interface in terms of the interacting atoms. Visualization of the interface and identification of the main interactions such as hydrogen bonding and hydrophobic interactions are displayed using the LIGPLOT system [19].

Inhibition of GPIb-vWf - mediated platelet agglutination

D-pep2, D-pep3 and D-pep4 were synthesized for laboratory experimentation. D-pep3, 10 mg, was synthesized by UBC's Peptide Proteomic Centre and was solubilized in Hepes-saline buffer, pH 7.4 and used at 0.1–0.5 mg/mL. Fresh washed platelets (160×10e8/mL) in Hepes buffer were added and agglutination was initiated by 1.25 mg/mL ristocetin (Sigma). Agglutination times and levels were monitored both microscopically and on an aggregometer (ChronoLog) as described by the manufacturer.

Results

The described methodology was applied to the set of peptides selected experimentally by their binding to purified vWf. Since the desired peptides should be oriented to inhibit the interaction between GPIb and vWf, the first step was the characterization of this interface.

Characterization of the interaction interface between vWf and GPIb

Figure 1 shows the complex and the interaction interface for the complex GPIb-vWf, as recorded in the Protein Data Bank (PDB) with the entry 1SQ0. Applying solvent accessible surface area (SASA) methodology to both units, using a water radius of 1.4 A, the result is shown in Fig. 2 where the interaction surfaces are mapped on each of the subunits constituting the complex GPIb-vWf (Fig. 2a: vWf, b: GPIb). The amino acids of the interfaces are listed below each figure. For an amino acid to be part of the interface, at least one of its constituent atoms is in contact with another atom of the interacting partner.

One of the most important properties driving proteins to interact with each other is the hydrophobicity of their surfaces. This physicochemical characteristic of the protein surface is usually expressed in terms of the number of hydrophobic amino acids present in particular regions of the molecular surface. Here, we performed a series of calculations in order to obtain these regions, using the SOM module in MIAX [11]. The learning steps were set to 6000, and the filtering coefficient was set to 5 [11]. The results are shown graphically in Fig. 2c and d together with the list of the amino acids composing the main hydrophobic region, for each of the components of the complex of GPIb and vWf.

A careful inspection of the list of amino acids of the hydrophobic patch on vWf (**K549**, **W550**, **S562**, H563, **Y565**, R571, I580, **E596**, **K599**, **Y600**, **P603**, **Q604**, **I605**, P606, S607, R611, E613, **R632**) with those involved in the interaction with GPIb: **K549**, **W550**, **S562**, **Y565**, **E596**, **K599**, **Y600**, **P603**, **Q604**, **I605**, **R632** (Fig. 2c & d) shows that all of the computed interactive amino acids are present in the hydrophobic patch (concordances in **bold**). Furthermore, experimental studies by Shimizu et al. [20] as well as those of Hauert et al. [21] established the importance of several of these amino acids by mutation assays that led to inhibition of the protein interaction between GPIb and vWf. They focus especially on amino acids R571, E613, K599 through P611 and R632, coinciding to a high degree with the computed results obtained here.

Selection of peptides that interact with vWf

As the identification of a vWf-binding, potentially antithrombotic agent was the objective of the search, D-amino acids, rather than L, were chosen for their ability to ultimately resist proteolytic cleavage in the mammalian circulation. Peptides on random 15-mer peptide arrays that were built of D-amino acids were selected on the basis of their ability to bind to vWf. Only four positive sequences were identified: D-pep1 - VSRQN G KQYW AIKEG; D-pep2 - WQNEG THVLS RCYEC; D-pep3 - RSARM QVCWN AFKNR; and D-pep4 - DSCPR DWDNN FLFFE. By definition, although their binding to vWf identified the peptides, the location of their attachment to the vWf molecule remained to be determined. Whether that binding site was at the vWf-GPIb interface, and thus could

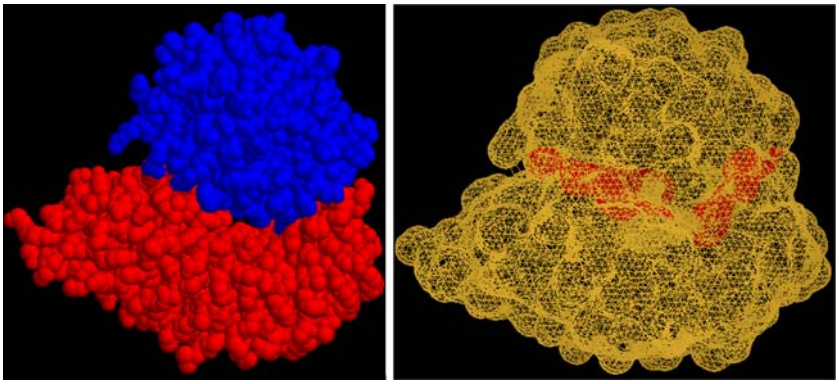

Fig. 1 **a**) Spacefill model of the vWf-GPIb complex. (vWf=blue; GPIb= red). **b**) Computed interaction interface of the vWf-PGIb complex

potentially inhibit the vWf-GPIb interaction was not known therefore the identification of each vWf - peptide binding interface constitutes the results that follow.

Modeling the 3D structures of the selected D-peptides

Three dimensional structures for the experimentally selected peptides are modeled according to the methodology described. Results for the four peptides are summarized in Table 1.

Figure 3a shows the MD simulation process for each one, while sketches of the structures as ribbon models are shown in Fig. 4. These are inferred structures based on the comparison of the peptides' linear sequence with similar L-amino acid sequences of natural proteins. The derived structures are then converted to D-amino acids and subjected to minimizations and MD simulations to derive the most energetically stable conformations of the peptides in solution.

In Table 1 the sequence of each peptide is shown together with the most similar sequence derived by a FASTA protein comparison search from PDB. The backbone of such a peptide was used as the starting backbone structure for each peptide before molecular dynamics simulation. Table 1 also summarizes the energies of the D-peptides after undergoing the conformation shift and the MD simulation process until energy convergence was achieved, as well as energies after minimization of the MD derived peptide structures, this procedure is performed in order to obtain the most realistic conformation for each peptide in solution.

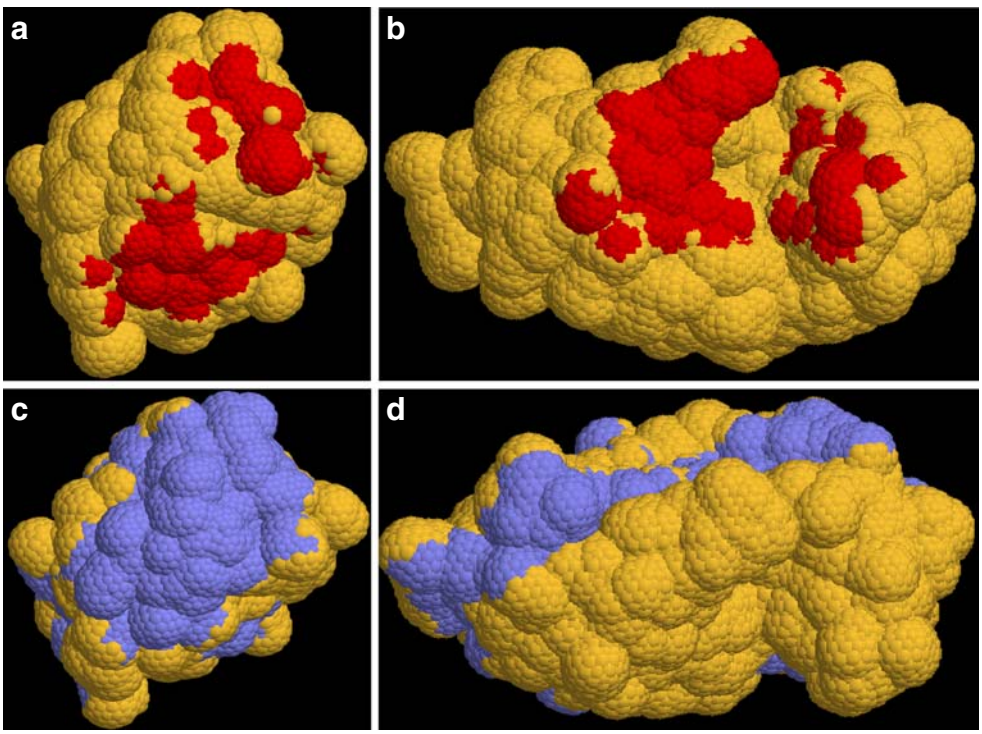

Fig. 2 Composition of the interaction interface of the GPIb-vWf complex (**a** & **b**) and the hydrophobic patches on the subunits of the GPIb-vWf complex (c & d). The amino acids of the interaction interface are: **a**) vWf: K549, W550, S562, Y565, E596, K599, Y600, P603, Q604, I605, R632; **b**) GPIb: V9, A10, K152, F199, E225, D235, V236, K237, M239, T240. The hydrophobic patches contain (**c**) vWf : K549, W550, S562, H563, Y565, R571, I580, E596, K599, Y600, P603, Q604, I605, P606, S607, R611, E613, R632; (**d**) GPIb: D21, T23, P27, D28, K31, L42, Y44, M52, P53, T55, E66, P77, V78, Q88, F109, R121, K137, T145, N157, E172, E181, S194, R218, D252, K253, K258, P260, K262

Table 1 Characteristics of the D-peptide conformation modeling process

PEPTIDES	**D-pep1**	**D-pep2**	**D-pep3**	**D-pep4**
Sequence	VSR QNG KQY WAI KEG	WQN EGT HVL SRC YEC	RSA RMQ VCW NAF KNR	DSC PRD WDN NFL FFE
FASTA output of most similar sequence	I50-G64 of PDB:1XSX	D141-C155 of PDB:1M8Y	R13-K27 of PDB:1W81	P31-L45 of PDB:1A88
Energy of Dpeptide after conformation change and MD (kcal/mol)	595.81	140.19	55.49	141.49
Energy of Dpeptide after minimization (kcal/mol)	−777.22	−397.87	−954.67	−895.43
COMPLEXES	**vWf-D-pep1**	**vWf-D-pep2**	**vWf-D-pep3**	**vWf-D-pep4**
Energy vWf (kcal/mol)	−3350.00	−3350.00	−3350.00	−3350.00
Energy Complex (kcal/mol)	−4870.12	−4220.2	−5110.2	−5460.31
BE (kcal/mol)	−742.90	−472.33	−805.53	−1124.88

Docking the peptides to vWf using MIAX

After modeling the 3D structure of the four D-peptides, the next step was to determine the peptides' binding sites on the vWf molecule by docking them in silico to the target vWf, using MIAX (*vide infra*). The complexes obtained by MIAX were submitted to further MD simulation and energy minimization to relax the structure (Fig. 3b).

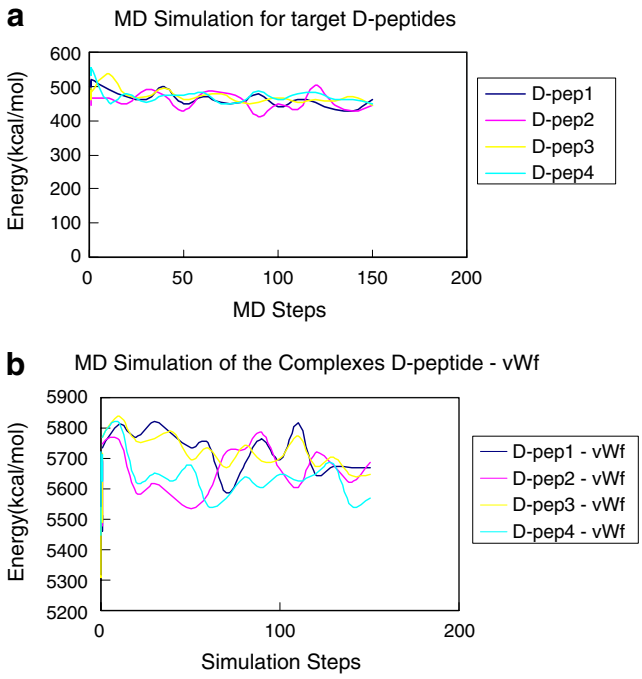

Fig. 3 Molecular dynamics simulations for the 4 target D-peptides (**a**) and the vWf-peptide complexes obtained by the docking experiment (**b**). To obtain the most energetically stable conformations for the peptides in solution a series of minimizations and MD simulations were carried out. All these computations were performed using the force fields in AMBER-6

Determination of inhibitory potential

Since the purpose was to block the protein-protein interaction between vWf and GPIb, we performed a further analysis of the interface of the GPIb-vWf complex. This additional analysis consisted of computing the entire network of hydrogen bonds and hydrophobic interactions that bind these two proteins. We carried out the computation using HYPLUS [22] which outputs the quantitative characteristics of the hydrogen bonds and LIGPLOT [19] for their visualization. This additional computation was aimed at enabling a comparison of the interfaces of the original complex and the peptide-vWf complexes obtained by docking (*vide infra*). Table 2 shows the inter-unit hydrogen bonds computed using the HYPLUS [22] system and summarizes the characteristics of the hydrogen bonds at the interface. The main characteristics shown are the polypeptide chains (A for vWf and B for GPIb), the number of the amino acids involved in the hydrogen bond as donor and acceptor, and the PDB names of the donor and acceptor atoms. Additionally, the donor - acceptor distance (D-A), the hydrogen acceptor (H-A), and the respective angles are also illustrated in Table 2.

Individual peptide docking results

Each docking experiment was performed in two stages. The first was the soft docking [9, see Methods], and the second consisted of performing molecular dynamics on each complex (vWf-peptide) to relax the structure and to evaluate the most important features of the complex output by MIAX. Figure 3b illustrates the MD simulation

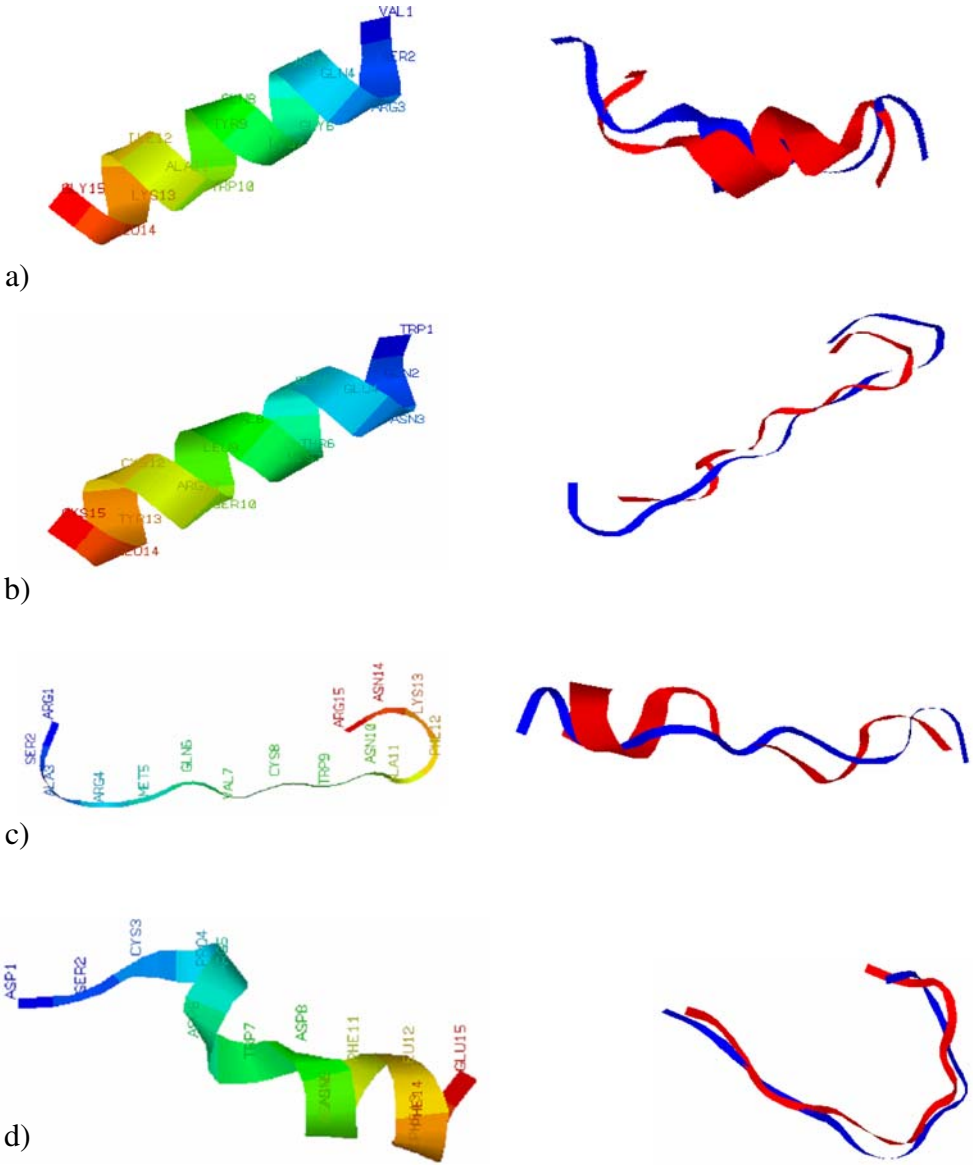

Fig. 4 Backbone models for the peptides under study. Starting conformation (left), Conformations after MD simulation (red) and point minimization (blue) for (**a**) D-pep1, (**b**) D-pep2, (**c**) D-pep3 and (**d**) D-pep4

for each of the complexes obtained by the docking experiment.

Table 3 shows the energies of the complexes after the energy minimization procedure. Binding energy (BE) calculated as:

$$BE = E(complex) - [E(vWf) + E(D - peptide)] \qquad (2)$$

was computed for each complex to evaluate the stability of the derived species.

A final evaluation of the complex output by the computational process described here was performed to characterize the complex in terms of the network of hydrogen bonds at the interaction interface as well as the hydrophobic interactions identified by means of the MIAX [11], HYPLUS [22] and LIGPLOT [19] software programs.

The soft docking module of MIAX [10] has the characteristic of optimizing the contacts among receptor and ligand atoms that may attract each other by electrostatic and London forces, and outputs a list of candidate conformations for the complex (decoys). MIAX does not a priori require specification of the binding site, however information on the interaction interface of any of the interacting subunits is valuable at the final ranking stage. The ranking of the decoys is then performed according to the scoring function that takes into account the energy of the complex, the geometric complementarity of the receptor and ligand as well as the a priori knowledge of 'hot spots' (which in this case are the hydrophobic patches on the surfaces of the receptor). Here we analyzed decoys that have been ranked high, and we performed an analysis of the forces that may lead

Table 2 Characteristics of the intermolecular hydrogen bonds of the vWf-GPIb complexes

Donor		Acceptor		Dist D-A	DHA[e]		Dist. H-A	Angles	
Amino Acid	Atom	Amino Acid	Atom		dist	angle		H-AAA	D-A-AA
[a]A0549[b]-LYS[c]	NZ[d]	B0005-GLU	OE1	3.32	11.79	170	2.33	99.8	100.2
A0562-SER	N	B0239-MET	O	2.91	5.39	160	1.95	146.8	150.2
B0239-MET	N	*A0562-SER	O	3.01	5.39	148	2.11	145.3	154.9
A0564-ALA	N	B0237-LYS	O	3.21	5.29	167	2.22	128.5	126.5
B0237-LYS	N	A0564-ALA	O	3.04	5.29	153	2.12	134.6	142.8
A0571-ARG	NE	B0018-ASP	OD2	2.91	9.38	166	1.93	138	134.9
*A0571-ARG	NH2	B0039-SER	OG	2.87	10.86	109	2.39	130.2	136.5
B0228-TYR	OH	*A0596-GLU	OE1	2.91	11.22	171	1.92	103.6	102.6
*A0599-LYS	NZ	B0198-PRO	O	3.13	8.6	157	2.19	123.5	123.7
A0599-LYS	NZ	B0228-TYR	OH 2.86	12.57	159	1.9	115.7	116.7	
B0152-LYS	NZ	A0603-PHE	O	3.01	9.7	157	2.06	127.9	133.8
*A0604-GLN	NE2	B0176-THR	OG1	2.85	8.54	164	1.87	147.2	145.4
*A0632-ARG	NH2	B0225-GLU	OE1	2.52	11.09	119	1.88	120.9	112.2

a) Subunit: A = vWf, B = GPIb
b) Amino acid number within the subunit
c) Amino acid name
d) Atom name
e) DHA (donor, hydrogen, acceptor)
*Homolog hydrogen bonds, found in the vWf-GPIb complex and in the vWf-peptide complexes below.

to vWf - D-peptide complex formation. We have mainly studied these aspects from the number of hydrogen bonds formed in the interface, and the stability of the complex expressed in terms of the binding energy (Eq. 2) resulting from the energy to which the MD run converges after a certain number of simulation steps and a further energy minimization process. This evaluation has been extended to compare the plausible hydrogen bonds in the interface of the predicted complexes with those in the experimental vWf-GPIb complex.

Complex of vWf and D-peptides

Figure 5 (a–d) and Table 3 summarize the characteristics of the peptide-vWf complexes. Table 3 summarizes the characteristics of the inter-molecular hydrogen bonds for each of the complexes and hydrogen bonds sharing homology with those of the original vWf-GPIb complex are marked with an asterisk.

For the first complex obtained by docking D-pep1 with vWf factor (vWf - D-pep1) Fig. 5a illustrates the position of the ligand peptide D-pep1 in the complex output as number one by MIAX, based on the scoring system as described above. The interaction can be quantified by the number of hydrogen bonds formed in the interaction interface (Table 3), where the amino acids holding the donor and acceptor atoms are listed together with the distances and angles of each hydrogen bond. Amino acids belonging to vWf are represented by chain A while amino acids of the peptide ligands are chain B in the table. Additionally, asterisks point to homolog hydrogen bonds observed in the wild type complex of vWf - GPIb. It is evident that vWf amino acids ARG571, SER562, GLN604, SER607, HIS563 and TYR565, play a critical role in the formation of this complex, although ARG571, SER607 and HIS563 are not directly involved in the vWf - GPIb interface as computed. The binding energy of the vWf - D-pep1 complex is −742.9 kcal/mol (Table 1).

For the second complex (vWf - D-pep2) Fig. 5b illustrates the position of the ligand peptide D-pep2 in the complex output as number one by MIAX. It is evident that in the case of the vWf - D-pep2 complex the amino acids of vWf ARG562, ARG599, ARG629, ARG632 and ASN633 play a critical role in the formation of the complex of which ASN633 and ARG629 were not in the computed vWf - GPIb interface (Fig. 2). The binding energy of the vWf - D-pep2 complex is 472.33 kcal/mol (Table 1).

For the third complex (vWf - D-pep3) Fig. 5c and Table 3 summarize the characteristics of the complex obtained by docking D-pep3 with vWf. In the case of the vWf - D-pep3 complex the amino acids A560 A563, play a critical role in the formation of the complex. Although neither of these amino acids is directly involved in the computed vWf - GPIb interface, the peptide sequence should have inhibitory activity as it binds to amino acids that are next to those involved in the interface. The binding energy of the vWf - D-pep3 complex is −805.53 kcal/mol (Table 1).

For the fourth complex (vWf - D-pep4) Fig. 5d and Table 3 summarize the characteristics of the complex

Table 3 Characteristics of the intermolecular hydrogen bonds for each of the vWf D-pep complexes

vWf-D-pep1									
Donor		Acceptor			DHA			Angles	
Amino Acid	A to m	Amino Acid	A to m	Dist D-A	dist	angle	DistH-A	H-A-AA	D-A-AA
0004-GLN	NE2	*A0562-SER	O	2.96	7	120	2.34	120.7	137.4
B0001-VAL	N	A0563-HIS	NE2	3.17	5.57	152	2.24	103.1	92.6
B0002-SER	OG	A0565-TYR	OH	3.29	7.14	172	2.31	92.2	93.4
*A0571-ARG	NH1	B0014-GLU	OE1	2.8	11.36	118	2.18	135.8	151.4
B0011-ALA	N	*A0604-GLN	O	2.99	4.47	129	2.22	114.7	120
B0007-LYS	NZ	*A0604-GLN	OE1	3.08	8.25	155	2.16	108.4	107.4
A0607-SER	N	B0015-GLY	OXT	2.92	4.47	172	1.93	117.6	114.8
A0608-LYS	NZ	B0014-GLU	O	3.11	7.94	121	2.47	150.7	146.6
A0616-ARG	NH1	B0015-GLY	O	3.01	11.31	145	2.1	128.8	129.8
vWf-D-pep2									
B0012-CYS	N	*A0562-SER	O	3.45	6.4	172.0	2.44	166.9	166.9
B0013-TYR	OH	A0599-LYS	O	2.96	10.0	142.2	2.15	109.0	106.9
*A0599-LYS	NZ	B0008-VAL	O	2.75	10.5	125.7	2.00	138.2	154.1
*A0599-LYS	NZ	B0009-LEU	O	3.31	8.83	135.5	2.48	111.0	123.8
A0629-ARG	NE	B0004-GLU	OE1	2.97	5.74	155.8	2.02	94.2	94.6
A0632-ARG	NE	B0002-GLN	O	3.00	6.93	159.5	2.03	140.7	144.5
*A0632-ARG	NH2	B0002-GLN	O	3.25	6.93	145.6	2.39	162.0	169.1
*A0632-ARG	NH2	B0003-ASN	OD1	3.28	6.4	142.3	2.44	146.3	156.0
A0633-ASN	ND2	B0004-GLU	O	3.18	7.75	141.3	2.34	100.5	111.8
vWf-D-pep3									
B0001-ARG	NE	A0560-ASP	OD1	2.83	7.62	138.9	1.97	115.9	106.2
B0008-CYS	SG	A0563-HIS	NE2	3.38	5.57	127.6	2.42	122.8	105.3
vWf-D-pep4									
B0005-ARG	NH2	*A0596-GLU	OE2	2.77	11.87	157.6	1.77	135.1	135.9
A0600-TYR	OH	B0015-GLU	OXT	2.66	9.7	158	1.73	157.6	150.3
A0629-ARG	NH2	B0008-ASP	OD1	3.03	8.12	143.7	2.15	134.5	123.5
A0637-TYR	OH	B0001-ASP	OD2	2.96	10.86	155.3	2.08	94.8	100.4
B0005-ARG	NH1	A0637-TYR	OH	3.46	13.45	173	2.44	123.8	122.8

f) Subunit: A = vWf, B = GPIb
g) Amino acid number within the subunit
h) Amino acid name
i) Atom name
j) DHA (donor, hydrogen, acceptor)
* Homolog hydrogen bonds, found in the vWf-GPIb complex and in the vWf-peptide complexes.

obtained by docking D-pep4 with vWf. In the case of the vWf - D-pep4 complex that the amino acids GLU 596, ARG629 and TYR637 play a critical role in the formation of the complex, and of them GLU596 is also involved in the originally computed vWf - GPIb binding interface. The binding energy of the vWf - D-pep4 complex is −1124.53 kcal/mol (Table 1).

Platelet inhibitory function of D-Peptides

Of the four selected peptides, three were synthesized for biological testing. We eliminated D-pep1 because of its lack of cysteine, an amino acid we required for further experimentation. As biological protein-platelet interactions need to be measured at physiological pH, in iso-osmotic buffers, D-pep2 and D-pep4 were also eliminated due to their lack of solubility at the pH range required for testing. However, the in vitro function of D-pep3 was confirmed by inhibition of ristocetin-mediated platelet agglutination, and quantitated by microscopy: control platelets agglutinated to ristocetin within 5 minutes but a 100-fold excess of peptide, based on plasma vWf content, prevented visible agglutination at 20 minutes (data not shown). Aggregometry confirmed dose-dependent inhibition of ristocetin-initiated agglutination of washed platelets such that at 10 minutes, bovine serum albumin at 0.5 mg/mL, buffer, and 0.1 mg/mL D-pep3 gave 55 %, 66 % and 69 % agglutination, while D-pep3 at 0.25 and 0.5 mg/mL reduced agglutination to 28 % and 13 % respectively. Similar patterns were observed for each of the

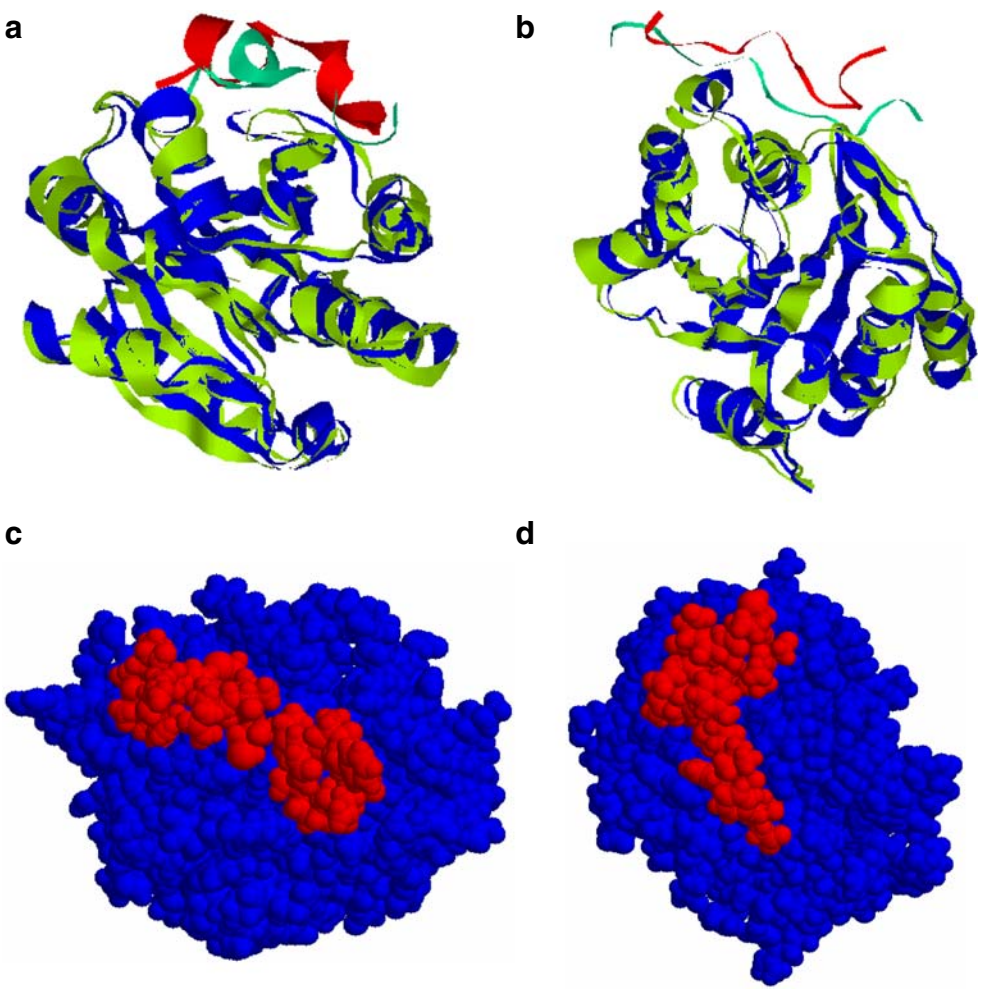

Fig. 5 MIAX derived complex of vWf and each of the 4 D peptides. The relaxation process for D-pep1 (**a**) and D-pep2 (**b**) is shown using molecular dynamics of the best decoy output by MIAX for this interaction. Initial and final configuration for the complex and the position of D-pep1 on the surface of vWf (teal) before and after (red) the molecular dynamics simulation. For brevity, space-fill models are shown for the complex vWf - D-pep3 (**c**) and D-pep4 (**d**)

individual blood donors tested confirming the platelet-inhibitory ability of D-pep3.

Discussion

Given a target (protein), designing a recognition molecule that interacts with it is intrinsically as difficult as predicting structural function as the number of possible protein structures is very large, and the physical basis of protein structural stability is incompletely understood. Consequqently any method that attempts to predict protein structure must access an array of possible structures as well as provide a means to identify the most plausible structure among them. An approach is to screen thousands of compounds for binding activity or inhibition of protein-protein interactions and thus search for potential drug candidates. This technique is suitable for computer design when the structure of the complex is available. Targeting protein-protein interactions, without knowledge of the 3D structure of the protein components requires identification of the key amino acids involved in that protein-protein interaction. Experimentally, this is done by point mutation experiments. Advances in crystallographic data analysis that allow the determination of protein complex structures, make it possible to design inhibitors to proteins using bioinformatic approaches by targeting the interaction sites between the subunits composing the complexes.

This computational study confirmed site-specific peptide-protein interactions by peptides that had been experimentally selected by their binding to vWf. The peptides' binding locations within the recognition domain on vWf were crucial to their ability to inhibit the protein-protein interaction between vWf and GPIb. Following screening of an 1120 peptide random array four peptides were selected by identifying them on the basis of their ability to bind to vWf. At this stage, the location of the peptide's binding site on vWf was unknown and therefore the potential of the peptide to interfere with the vWf-GPIb interaction remained to be determined. Evaluation of the peptides as potential inhibitors of the interaction between GPIb and vWf consisted of using bioinformatics systems to assign three dimensional

structures to the peptides and to describe their potential spatial relationships with vWf. Three dimensional structures for the peptides were modeled using homology studies, to obtain an initial conformation for the D-peptides, and molecular dynamics and energy minimization processes were used to obtain the optimal 3D structures for each peptide. These structures were docked to their prospective binding partner, vWf, by means of the flexible docking module of MIAX. Since MIAX outputs a large number of decoys (>4000) ranked by geometrical and energy instances (geometrical complementarity and interaction energy), we selected only the best decoys for each of the four studies corresponding to the four peptides initially selected. These complexes were further relaxed by MD simulations.

Interfaces of the final vWf - D-peptide complexes were then evaluated for hydrogen bonding networks and hydrophobic interactions. Binding energy results show that D-pep4 binds to the vWf molecule with the highest affinity, followed by D-pep3, then D-pep1 and finally D-pep2. However, D-pep2 can form far more hydrogen bonds with VWf than the other three peptides. Many of the hydrogen bonds realized by docking D-pep2 to vWf share homology to the hydrogen bonds found in the original protein-protein complex (vWf - GPIb). The number of similar hydrogen bonds that D-pep2 is able to make with vWf in the best decoy output by MIAX is five while D-pep1 is able to make only four bonds, D-pep4 one and D-pep3 none. Stabilities of the complexes output by MIAX, signaled by the MD simulation, show that vWf - D-pep4 is the most stable, followed by vWf - D-pep3, then vWf - D-pep1, with the least stable being again vWf - D-pep2.

To summarize, D-pep4 may interact with the highest affinity and interaction energy to vWf followed by D-pep3 and D-pep1, while D-pep2 is the lowest ranked. Thus D-pep4 binding would be the most likely structure to interfere with the formation of the GPIb-vWf complex and would constitute a promising inhibitory peptide. However, this is where in silico methods defer to biological methods such that peptide solubility in physiological buffers affects the practical experimental choices that are made subsequently. As a result, only D-pep3 was available for testing with living cells.

Still, confirmation of the applicability of this process was done by synthesis of D-pep3, which was chosen because of its solubility in physiological buffers. Its ability to prevent vWf-mediated platelet agglutination in a dose-dependent manner functionally validated the in silico process.

Summary

In conclusion, we have used in silico methods to describe the L amino acids involved in the interaction interface between GPIb and vWf. Our calculations confirm and expand experimental findings [20, 21] that define the contributions of some of these amino acids to the binding site. We have also selected, then described, the binding sites of D peptides with antithrombotic potential by their inhibition of the GPIb-vWf interaction. Finally, by demonstrating inhibition of vWf-mediated platelet agglutination by D-pep3, we validated this in silico approach to the identification of antithrombotic as well as other potential drugs.

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