

Cite this: *Chem. Commun.*, 2012, **48**, 11349–11351

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COMMUNICATION

An integrated fragment based screening approach for the discovery of small molecule modulators of the VWF–GPIIb/IIIa interaction†

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Received 21st July 2012, Accepted 1st October 2012

DOI: 10.1039/c2cc35269a

An integrated approach comprising STD NMR screening, pharmacophore based analogue selection and a bioassay is presented for the discovery of a stabilizer of the clinically relevant VWF–GPIIb/IIIa protein–protein interaction.

Protein–protein interactions (PPIs) play key roles in nearly all biological processes. Since many disease-related pathways are influenced by PPIs, targeting PPIs using Small Molecule Protein–Protein Interaction Inhibitors (SMPPPIs) opens a pipeline for the discovery of new classes of drugs.¹ In recent years, considerable progress has been made in the design and discovery of SMPPPIs.^{2–5} The binding of the von Willebrand factor (VWF) to the glycoprotein (GP) IIb/IIIa receptor on blood platelets is a PPI of particular importance in maintaining haemostasis. Upon vascular injury, circulating plasma VWF adheres to subendothelial collagen through interactions with its A3 domain, which under high shear conditions induce conformational changes in VWF leading to unmasking of the cryptic binding site for GPIIb/IIIa within the VWF-A1 domain. The VWF–GPIIb/IIIa interaction allows initial tethering of blood platelets and the formation of a haemostatic plug that prevents excessive blood loss.⁶ Lowered levels of VWF result in a bleeding disorder known as von Willebrand's disease (VWD), whereas high VWF levels increase the risk of acute coronary syndromes and ischemic stroke,^{7,8} further underlining

the importance of VWF. To date two inhibitors of the VWF–GPIIb/IIIa interaction *i.e.* the nanobody ALX-0081⁹ and the aptamer 1779,¹⁰ both targeting the VWF-A1 domain, are under clinical development. However their size and/or peptide nature hamper oral administration, which is desirable for VWF-associated pathologies that require long term or prophylactic treatment.

We therefore aimed at identifying small molecule modulators of the VWF-A1–GPIIb/IIIa interaction using a fragment-based drug discovery (FBDD)^{11,12} approach.⁴ Usually, in a FBDD approach, fragments¹³ are screened to select low affinity binders ($K_d = \mu\text{M}$ to mM) against the target of interest using biophysical techniques such as NMR spectroscopy, X-ray crystallography and surface plasmon resonance. The compounds identified in this way can then be turned into a suitable drug candidate through fragment evolution, fragment growing and linking¹⁴ using additional information such as a known ligand, X-ray crystallography data of fragments bound to the target protein or SAR by NMR¹⁵ (structure–activity relationship by Nuclear Magnetic Resonance).

We set out to determine whether it would be possible to use data from STD NMR¹⁶ (Saturation Transfer Difference NMR) screening to identify small molecule modulators of the VWF-A1–GPIIb/IIIa interaction without the knowledge of small molecule competitive inhibitors that would normally identify the binding site. To this end, we selected VWF-A1-specific binding fragments by screening them in parallel with the homologous VWF-A3 domain¹⁷ under identical experimental conditions. For initial screening a diverse set of 80 molecules was selected from the Maybridge Ro3 500 fragment library. Criteria used were diversity in molecular shape¹⁸ and in chemical functionality. Molecules were subdivided into subsets of compatible functionality for screening as mixtures.

After screening of the fragments with VWF-A1¹⁹ using STD NMR, the STD amplification factor (A_{STD})²⁰ was calculated to rank the fragments. To have a criterion to distinguish the hits from non-hits, a lower cut off value of 1% (corresponding to the STD amplification factor of the TSP-*d*₄ (3-(trimethylsilyl)propionic-2,2,3,3-*d*₄ acid sodium salt) reference signal²¹ was taken into account, resulting in an overall hit rate of 30% (Table S1, ESI†). It should be mentioned that no large variations in the A_{STD} values were observed for the different fragments which makes it difficult to rank compounds.²⁰ Since neither the full NMR-assignment of the VWF-A1 domain nor

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2cc35269a

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a small molecule inhibitor is available, it was impossible to proceed with two-dimensional ^1H - ^{15}N or ^1H - ^{13}C NMR experiments or competition NMR experiments to select a suitable fragment for further development. Hence the fragment screening was repeated with the homologous VWF-A3 domain, the idea being that fragments also binding to the VWF-A3 domain were not interacting with a specific site of the VWF-A1 domain. Again, the fragment A_{STD} values were determined and evaluated with respect to the results of the VWF-A1 screening (Table S1, ESI†). Binding was observed for most of the fragments screened against VWF-A3 (usually with higher A_{STD} values as compared to VWF-A1), except for 2 fragments (**F1** and **F2** in Table 1). As we hypothesized that this was a good indication for a specific binding event between the two fragments and VWF-A1, we further explored this observation through pharmacophore based selection of analogues (**F3**–**F13** in Table 1) as described in the ESI.†

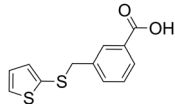
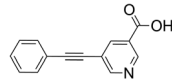
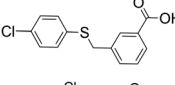
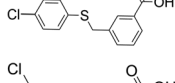
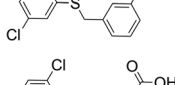
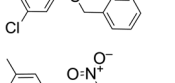
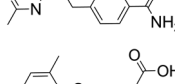
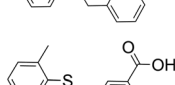
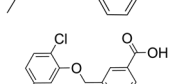
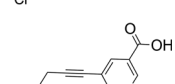
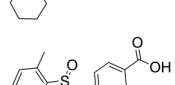
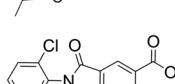
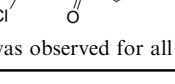
The selected fragments were further screened with VWF-A1 using STD NMR, and most of them showed an STD effect with VWF-A1. One of the compounds (**F6** in Table 1) even showed a marked line broadening in the ^1H NMR spectrum (ESI,† Fig. S2B).

We next investigated the influence of the fragments on the VWF–GPIIb α binding in an assay known as a ristocetin co-factor ELISA²² (see ESI†). In contrast to the original fragments **F1** and **F2** (which would have been missed if only a bioassay would have been performed), some fragments significantly increased the amount of VWF bound to GPIIb α at a concentration of 400 μM . The most pronounced effect was observed for fragment **F6** reaching up to $188 \pm 7.9\%$ (with $n = 3$ and $p < 0.001$) of the normal binding. Full solubility of the fragment at this concentration was confirmed by light transmission and NMR studies (data not shown).

As proton or STD NMR data suggested specific binding of the fragments to VWF-A1, the binding of VWF-A3 to collagen, should not be influenced, which was confirmed in a collagen binding ELISA²³ for all compounds (data not shown). In order to further explore the specificity of **F6** for VWF-A1, the binding of **F6** to GPIIb α ^{19,22} was tested using NMR. No line broadening was observed for **F6** in the presence of GPIIb α (Fig. S2C, ESI†), although an STD effect was observed, likely pointing out that **F6** is binding to VWF-A1 with higher affinity.

To rationalise the binding mode and the mechanism of action of the fragments that increase the binding of VWF-A1 to GPIIb α , molecular docking simulations were performed. The VWF-A1 structure was retrieved from the VWF-A1–GPIIb α co-crystal structure (PDB entry 1M10²⁴) and subjected to binding site analysis. The hotpatch algorithm²⁵ as well as the FT-map algorithm²⁶ indicated the presence of one (identical) site in the VWF-A1 domain able to accommodate small molecules. Using GOLD²⁷ the fragments were docked inside this pocket in the 1M10 VWF-A1–GPIIb α complex structure, as well as in the retrieved VWF-A1 domain alone. Analysis of the docked results revealed a common binding mode between different ligands in the 2 different docking experiments. Structural analysis of the complexes indicated that the hydrophobic core of the fragments is bound into the cavity, while the acid functionality creates an additional salt bridge with the Lys-237 of GPIIb α (Fig. 1). As such, the fragments may serve as a cofactor of the VWF-A1 domain and stabilize the

Table 1 The effect of the fragments on the VWF–GPIIb α interaction as determined in a ristocetin co-factor ELISA^a

Compound	Structure	Normalized % VWF binding ($n = 3$)	SEM	p Value
F1		104	2.2	NS
F2		110	3.3	NS
F3		154	1.3	0.007
F4		145	3.1	0.003
F5		129	5.7	0.036
F6		188	7.9	0.001
F7		108	3.2	NS
F8		133	4.1	0.033
F9		97.6	5.0	NS
F10		143	4.7	0.001
F11		131	10	0.034
F12		109	7.0	NS
F13		97.6	5.0	NS

^a STD effect was observed for all fragments shown in the table.

VWF-A1–GPIIb α complex formation by the introduction of an additional salt bridge.

Molecular modelling gives a clue towards the binding mode of the compounds to VWF-A1. It seems to explain the stimulatory mechanism by creation of an additional salt bridge and also explains the observed SAR for the derivate compounds. Although binding could be observed using STD NMR (Table 1), **F7**, **F9**, **F12** and **F13** do not exhibit a stimulatory effect. This agrees with the observed binding mode; **F7** lacks the required salt bridge forming acid functionality, and **F12** and **F13** have

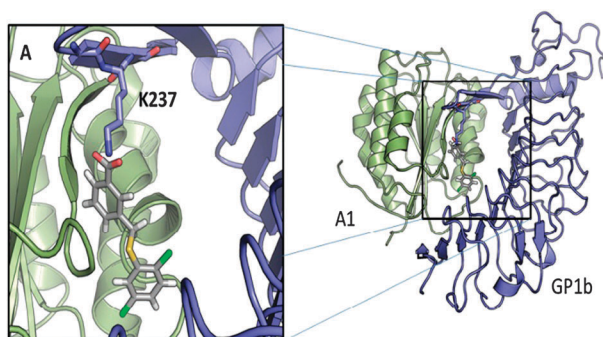


Fig. 1 Molecular docking simulations revealed a putative binding that explains the stimulatory mechanism of action of the fragment compounds. The hydrophobic core of the fragment molecule **F6** is buried in a superficial cavity while the acid group binds the Lys-237 of the GPIIb protein. As such the fragment serves as an A1 bound cofactor to promote and stabilize the binding of the GPIIb protein to VWF-A1.

different linkers, which make it impossible to accommodate a similar active conformation. Furthermore chlorinated phenyl substituents (**F3**, **F4**, **F5**, **F6** and **F10**) appear to be preferred over methylated substituents (**F8** and **F9**). This can possibly be explained by the observation that polarizable hydrophobic groups make favourable interactions with the protein surface which is hallmarked by the presence of adjacent polar amino acids. These phenyl decorations are clearly important contributors to the activity: the initial fragments did not have any and also did not exhibit any biological effect. Furthermore the chloride position seems to be of importance. Clearly, chloride is preferred at the *meta* position compared to the linker while any substituent (chloride or methyl) can be accommodated at the *ortho* position. This agrees with molecular modelling where the *meta* substituent is deeply buried in a pocket (lined by the hydrophobic part of polar amino acids) while the *ortho* position is semi solvent exposed. Substituents at other positions are less preferred, which agrees with the shape of the pocket according to molecular modelling. Keeping these insights in mind, new and putatively more potent derivatives can be designed in the future.

Interestingly, FBDD through STD NMR on two homologous protein domains has resulted in defining small molecules that actually stabilize, if not to say stimulate, the binding between two protein partners VWF and GPIIb. Recently Thiel *et al.*²⁸ reviewed the stabilization of PPI by small molecules, which they conclude to be an interesting target for drug discovery. However, previous successes in this field are based on PPI-stabilizing natural products. In extension of this observation we believe our results indicate that such molecules can also be identified using a rational fragment based method, validating this approach for this emerging class of targets. Although an inhibitory compound for the VWF–GPIIb interaction would have a broader clinical relevance, a compound enhancing the binding of VWF to GPIIb might be of interest for some patients suffering from von Willebrand disease (VWD) type I (see ESI†) that encounter severe bleedings *e.g.* in the case of surgery due to lowered levels of plasma VWF.

In conclusion, starting with NMR screening of a small commercially available library of in total about 80 fragments, we were able to identify compounds specifically binding to the VWF-A1 domain that modulate the VWF-A1–GPIIb PPI. This further illustrates that the results from STD NMR

screening can be used as a starting point for finding specific compounds even without having access to protein NMR-assignment data or a known reference small molecule for competition experiments. While the activity of the compounds remains in the higher micromolar range, it should be noted that no further optimisation has been performed after the second round of screening. Moreover the structural analysis of the binding of the compounds to VWF-A1 reveals the possibility to expand the compounds in the direction of GPIIb causing steric hindrance and as such evolving the molecular glue like fragments into fully active competitive SMPPIs of the clinically relevant VWF–GPIIb interaction.

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