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Overcoming challenges in developing small molecule inhibitors for GPVI and CLEC-2

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Abstract

~~Antiplatelet drugs are used for long term prevention of arterial thrombosis, yet do not work in all patients and have an intrinsic risk of increasing haemorrhage, limiting their clinical benefits. Within the past few years, increasing recognition of the role of platelets in inflammation-driven thrombosis, called thrombo-inflammation, has led to the identification of new targets that are implicated in these pathways. The platelet receptors~~ GPVI and CLEC-2 have emerged as promising targets for long-term prevention of both arterial thrombosis and thrombo-inflammation with a decreased bleeding risk relative to current drugs. However, while there are potent blocking antibodies of both receptors, their protein nature comes with decreased bioavailability, making formulation for oral medication challenging. Small molecules are able to overcome these limitations, but there are many challenges in developing antagonists of nanomolar potency, which is necessary when considering ~~the structural features that underlie the interaction of CLEC-2 and GPVI with their protein ligands.~~ In this review, we describe current small molecule inhibitors for both receptors and strategies to overcome such limitations, including considerations when it comes to *in silico* drug design and ~~the importance of complex~~ compound library selection.

Introduction

~~The platelet glycoproteins, GPVI and CLEC-2, are receptors for collagen and podoplanin, respectively, and are recognised as candidates for a new class of antiplatelet agent with a lower risk of bleeding relative to current therapeutics. Neither receptor is critical for haemostasis and both are implicated in platelet-driven thrombosis and thrombo-inflammation [1–3]. For example, GPVI has been shown to be directly involved in inflammation-induced thrombus and plaque formation leading to stroke and myocardial infarction[1–5]. GPVI is exclusively expressed in platelets and megakaryocytes, and~~

CLEC-2 is expressed on platelets and at low level on a sub-population of myeloid cells[6–9], suggesting that targeting either receptor is unlikely to induce significant off-target effects. This is supported by the observation that genetic loss of GPVI in humans is well tolerated and associated only with a mild bleeding diathesis[10–13]. Further, although humans deficient in CLEC-2 have not been described[14], post-development genetic loss or antibody-induced deletion of CLEC-2 in mice has a minor or negligible effect on haemostasis and is also well tolerated [1]. Recent reviews[15–17], and two articles in this series[18, 19], provide further information on the role of the two receptors in arterial thrombosis and thrombo-inflammation.

Blocking antibodies and related biologics exist for both receptors and many have nanomolar affinity [20–23], as the one under clinical trial, ACT-017, binding to the collagen binding site [24, 25], but they lack oral bioavailability and are expensive to produce. The development of small molecule inhibitors for long term targeting of GPVI and CLEC-2 however is highly challenging [26]. In general, protein ligands occupy a large surface area of the receptor, whilst small molecules by their nature interact with a smaller area [27]. In addition, many sites of protein-protein interactions (PPI) are relatively flat and lack a clear binding pocket, as is the case for GPVI [28]. As a result, the affinity of many PPI small molecule inhibitors falls in the micromolar range, including those currently identified for GPVI and CLEC-2 [28]. This review aims to highlights the methodologies and techniques that need to be employed to bridge the gap between proteins and **potent** small molecules inhibitors of GPVI and CLEC-2.

GPVI and CLEC-2 in haemostasis and thrombosis

~~GPVI and CLEC-2 are expressed in platelets and megakaryocytes, while CLEC-2 is also expressed at low level on a sub-population of myeloid cells[6–9]. Neither receptor is critical for haemostasis. The genetic loss of GPVI in humans abrogates platelet activation~~

by collagen but does not abolish adhesion and is associated with a mild bleeding diathesis[10–13]. Patients deficient in CLEC-2 have not been described[14], and the constitutive or antibody-induced deletion of CLEC-2 in mice has a minor or negligible effect on haemostasis as determined by measurement of tail bleeding. CLEC-2 and GPVI are involved in platelet-driven thrombosis and thrombo-inflammation[1–3]. GPVI has been shown to be directly involved in inflammation-induced thrombus and plaque formation leading to stroke and myocardial infarction[1–5].

Molecular basis of platelet activation by GPVI and CLEC-2

The clustering of GPVI and CLEC-2 drives intracellular signalling cascades that leads to activation of platelets. GPVI is a single transmembrane protein belonging to the immunoglobulin family of receptors that, the transmembrane domain is expressed in the membrane with the dimeric Fc receptor (FcR) γ -chain, with each chain having an immunoreceptor tyrosine-based activation motif (ITAM), characterised by two conserved YxxL sequences[29]. In contrast, the single transmembrane, lectin-like receptor, CLEC-2, has one YxxL sequence in its cytosolic tail, named a hemITAM (or hemi-ITAM)[30]. Clustering of GPVI or CLEC-2 leads to phosphorylation of the conserved tyrosines in the hemITAM or ITAM sequence by Src and Syk tyrosine kinases, leading to binding of the tandem SH2 domains in Syk and initiation of a downstream signalling cascade orchestrated through the protein adapter LAT. This acts as a binding template for other proteins facilitating a phosphorylation cascade, including various adapter and effector proteins, leading to activation of PI 3-kinase and PLC γ 2 (Figure 1). PI 3-kinase generates the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) which binds to pleckstrin homology and SH2 domains. PLC γ 2 generates the second messenger inositol 1,4, 5-trisphosphate (IP3) and 1,2-diacylglycerol, which release Ca²⁺ and activate protein kinase C, respectively.

GPVI and CLEC-2 ligands

Collagen is generally considered to be the ‘major’ endogenous ligand of GPVI. It binds to GPVI through multiple glycine–proline–hydroxyproline (GPO) motifs thereby causing receptor dimerization and higher order clustering[31–33]. Fibrin and fibrinogen have also been shown to activate GPVI, although it is controversial whether this is through direct binding as the site of interaction has not been mapped [34]. The collagen mimetic, collagen related peptide (CRP), composed of GPO repeats and crosslinked by C- and N-terminal lysines or cysteines, and the snake venom toxin, convulxin, are commonly used to activate GPVI due to their potency and absence of binding to a second receptor for collagen on platelets, integrin $\alpha 2\beta 1$ [11, 35–37]. **The binding site of convulxin on GPVI is distinct from that of collagen[37].**

Podoplanin is considered to be the major endogenous ligand for CLEC-2, although heme, **which is released** from damaged red blood cells, has also been shown to activate the C-type lectin-like receptor and this may be relevant in haemolytic disease[38]. Podoplanin is widely distributed on lymphatic endothelium[39], kidney podocytes[40] and various epithelial membranes, including in the lung and choroid plexus, and is up-regulated on haematopoietic cells at sites of inflammation[41]. It is also expressed on a variety of tumour cells[42]. The snake venom toxin rhodocytin, which is an $(\alpha\beta)_2$ -tetramer constituted by two α - and two β -subunits, also induces powerful activation **of** CLEC-2. ~~**Rhodocytin was used as an affinity ligand in the original identification of CLEC-2 in platelets[43].**~~—The binding site for rhodocytin on CLEC-2 overlaps with that for podoplanin, with both ligands binding to four conserved arginine residues located as well as to a second, non-overlapping site[44, 45]. The binding site is located on the opposite side to the carbohydrate-like recognition region in CLEC-2.

The challenge in developing protein and small molecule inhibitors of GPVI and CLEC-2

The clustering of GPVI and CLEC-2 is achieved either through the presence of repeat sequences **on a single ligand (i.e. collagen)** or the accumulation of individual ligands in the membrane (i.e. podoplanin). Inhibition of such systems is traditionally targeted through antibodies and related biologics: agents characterised by high affinity and slow off-rates, with binding further influenced **by avidity** due to their dimeric nature. However, antibodies or biologics agents are not ideal for long-term prevention for patients at risk of thrombosis as they are not orally available.

The interactions between a protein ligand and a receptor can be difficult to mimic in a small molecule as the site of interaction may have relatively few features and cover a large surface area. This is depicted in Figure 2 in which a small monomeric is more appropriate molecule is shown to reduce but not block binding of a ligand. Ideally, a small molecule ligand should bind to a key site in the receptors with high affinity and have a slow off-rate to counter the effects of avidity. Therefore, identifying key target residues is crucial to drug design and more complex molecules are likely to be more powerful inhibitors. As such, the use of more complex libraries during screening, such as natural compound libraries, is more appropriate as **the ligand may interact with the receptor at more than one site (Figure 2).** Alternatively, small molecules may inhibit at an allosteric site, thus causing a conformational change in the ligand binding site, or prevent receptor clustering as shown in Figure 2. Many proteins however undergo minimal change in conformation on ligand binding and have large areas that support clustering, with often no critical features. These considerations also apply to small molecule irreversible inhibitors, **although these have the advantage of countering the avidity** [46]. A number of additional factors should also be taken into account during drug design, including protein half-life, binding site surface area, target residue identification, chemical group reactivity

and reversible ligand selection, while being backed up thoroughly by structural data, are required to engineer potent irreversible ligands [47].

Development of orally available RGD mimetics

One successful example of developing small molecules that block the interaction of a protein ligand against its receptor is that of RGD (Arg-Gly-Asp) mimetics which block binding of multiple protein ligands to integrins. Several of the ligands for integrin $\alpha\text{IIb}\beta 3$, including von Willebrand factor and fibronectin, have a common tripeptide motif RGD that interacts with a distinct epitope in the integrin. One exception to this is fibrinogen which binds to the same epitope but through its terminal γC domain[48]. RGD mimetics can selectively block the interaction of the major platelet integrin, $\alpha\text{IIb}\beta 3$, with many of its ligands. The RGD residues bind to the β -propeller in the α subunit and the A-domain in the β subunit and coordinate the divalent cation to elicit conformational changes of the receptor and subsequent activation [49, 50].

The RGD motif has inspired the development of RGD mimetics to contain bioisosteres of endogenous components of the RGD sequence, with enhanced physicochemical properties and re-engineered conformation. The common short peptide RGD and the conformational flexibility of the RGD motif determines affinity and integrin sub-type selectivity. The development of RGD mimetics has also benefitted from the discovery of RGD-containing natural peptides, which later allowed the cyclization of RGD-containing peptides to increase the affinity and selectivity. Sequences with slight variations in the RGD-motif have also been successful, such as the cyclic heptapeptide, eptifibatide, derived from a snake venom with a Lys-Gly-Asp (KGD) sequence[51]. Non-peptide, small molecule inhibitors based on the RGD motif have also been developed. Chemical optimization of known RGD-mimetics has led to the discovery of other structures with

comparable potency, such as tirofiban with tyrosine as a backbone[52]. Eptifibatide and tirofiban are used in the clinic for the treatment of acute thrombosis[52, 53]. Advances on chemical optimization has also led the design of prodrugs that are orally available. Lotrafiban is an orally available RGD mimetic prodrug that reached phase III of clinical trials, but did not proceed further due to excessive bleeding[54, 55]. Nevertheless, this demonstrates that it is possible to develop potent, orally available inhibitors against PPIs, at least when there is a major epitope in the receptor.

GPVI structure and ligand interactions

The crystal structure of GPVI, and more recently the structure bound to the collagen mimetic, CRP, has been reported (Figure 3). This extends earlier mutational and molecular modelling studies and has revealed the key amino acids that affect collagen and/or CRP binding.

Mutations in amino acids that affect CRP and collagen binding were reported over ten years ago [56, 57]. Mutational studies highlight amino acids that either bind to collagen and/or CRP, which cause a conformational change in the binding site. Mutational studies identified four amino acids (Gly30, Val34, Leu36 and Arg38) that influence the binding of CRP and collagen to GPVI ~~and are potential binding site stabilizers~~[37]. Three of these are extensions of the ligand (orthosteric) binding site and Arg38 forms part of the binding site [37, 58] (Figure 3). A fifth amino acid, Asp72, the site of N-glycosylation, is located close to the binding site[59] but does not appear to play a role in ligand binding as it was mutated in the crystal structure of the CRP-GPVI complex. Mutation of Lys59, Arg60 and Arg166 decreases the affinity for binding to CRP and collagen. These three amino acids are located towards the GPVI ectodomain dimer interface and away from the CRP binding site where they form a linear orientation in the D1 domain demonstrating this is a site of allosteric interaction (Figure 3). The mapping of the CRP binding site by co-

crystallisation of GPO-3 and GPO-5 repetition peptides with the GPVI D1 and D2 ectodomains (PDB files: 5OU8, 5OU9) has shown a direct interaction with Glu40, Arg67, Gln71 and Trp76, alongside Arg38 (Figure 3) and is in agreement with previous mutational and molecular modelling studies[59].

In summary, many of the amino acids identified in the early mutagenesis studies are now recognised as distinct from the binding site for CRP suggesting that these may be targets for allosteric modification. The mapping of the CRP binding site reveals multiple points of contact with GPVI on a relatively flat surface thereby making it challenging to identify a small molecule inhibitor.

CLEC-2 structure and ligand interactions

The crystal structures of CLEC-2 have shown that podoplanin and rhodocytin bind to a common site to induce activation, constituted by four arginine residues (Arg107, Arg118, Arg152, Arg157), together with distinct, adjacent sites which act through polar interactions[44, 60] (Figure 4). Podoplanin binds to CLEC-2 through a series of conserved sequences known as a platelet aggregation stimulating (PLAG) domain. Four PLAG domains have been identified in human podoplanin which are characterised by the sequence ED-(X)XX-T. The O-glycosylation site, Thr52 within the human PLAG3 domain, has a sialic acid residue which is critical for binding[43, 44]. In addition, mutagenesis of the PLAG4 domain suppresses the CLEC-2-podoplanin interaction, demonstrating the importance of this region[44, 61] (Figure 4).

The crystal structure of CLEC-2 in complex with its exogenous ligand rhodocytin revealed that the negatively charged residues Glu3, Asp4, and Asp6 in the α -subunit of rhodocytin form electrostatic interactions with the positively charged residues of Arg107,

Arg118, Arg152, Arg157 in CLEC-2[60, 62]. Carboxyl groups on the C-terminus of rhodocytin also interact with CLEC-2 based on polar contacts (Figure 4).

Small molecule inhibitors of GPVI

Several small molecule GPVI inhibitors have been reported which are shown in Figure 5A. The most thoroughly studied of these is the angiotensin II receptor antagonist, losartan [63], which was found in a drug-repurposing virtual screening assay. Several natural bioactive compounds, namely honokiol [64], hinokitiol [65] and caffeic acid phenethyl ester (CAPE) have also been identified[66]. Honokiol is the only one of these for which direct binding has been studied using surface plasmon resonance [64]. As yet, molecular docking studies have not been reported with any of the compounds, and the value of these would be limited due to the low affinities of the compounds. S002-333[67], a pyridoindole based compound, and S007-867[68], a chiral 3-aminomethylpiperidine analogue, have also been shown to block GPVI and to have antithrombotic efficacy in animal models. All of the ligands (Figure 5A) display IC_{50} values in the micromolar range on platelet aggregation assays. Moreover, losartan has been shown to inhibit other platelet receptors, notably those for thromboxane A_2 and CLEC-2 at similar or slightly higher concentrations [69], which suggests that it may be having a generalised effect on membrane organisation rather than competing at the ligand binding site.

Small molecule inhibitors of CLEC-2

Cobalt-hematoporphyrin (Co-HP; Figure 5B) is a non-peptide inhibitor of CLEC-2-podoplanin interaction identified through a high throughput screening (HTS) assay and by chemical optimization of the lead compound [70]. The basis of the screening assay was an ELISA-based assay which detects loss of ligand-receptor binding. Co-HP was shown to inhibit aggregation of human and mouse platelets by podoplanin-expressing

cells and by rhodocytin at low micromolar concentrations. It has no effect on aggregation induced by thrombin or collagen[70]. Molecular docking predictions and site-directed mutations of CLEC-2 suggest that Co-HP binds to Asn120, Asn210 and Lys211[70] suggesting that inhibition is mediated by an allosteric action, since these amino acids are distant to the podoplanin binding site (Figure 4). Earlier, Chang *et al* (2015) had characterised 2CP[71] (Figure 5B), a small molecule able to inhibit aggregation induced by podoplanin at high micromolar concentrations, but which did not affect aggregation induced by rhodocytin, thrombin, collagen, or ADP. Surface plasmon resonance was used to show direct binding to CLEC-2 receptor with a K_d of 33 μ M. Molecular docking studies suggest that 2CP binds to the same canonical binding site of podoplanin and rhodocytin[71], although this is not consistent with the observation that 2CP inhibits podoplanin-induced but not rhodocytin-induced aggregation. It is therefore more likely that 2CP is competing for the non-canonical binding site of podoplanin on CLEC-2 which does not interact with rhodocytin. The binding site of 2CP needs to be mapped to resolve this question. CoHP has been shown to prolong occlusion in a $FeCl_3$ injury model[70] but the low affinity, lack of oral availability and toxicity of both compounds means that it is not a candidate for clinical development.

It may be possible to design small molecules based on the binding site of antibodies to CLEC-2. The only current monoclonal antibody to human CLEC-2, AYP1, has been shown to compete with rhodocytin and podoplanin for binding to the receptor, suggesting that it binds at the ligand binding site but, as yet, the site of interaction has not been mapped [23]. The crystallisation of AYP1 with CLEC-2 will provide further information on key residues for development of small molecule inhibitors.

Approaches to identify novel inhibitors against CLEC-2 and GPVI

Computational modelling

Molecular modelling and virtual experimentation are powerful tools ~~that help us in~~ screening and design of ligands with enhanced selectivity and physicochemical properties (Figure 6). The structure of a protein binding site can be obtained by x-ray crystallography, cryogenic electron microscopy (cryo-EM), multi-dimensional nuclear magnetic resonance (NMR) and molecular simulations (or homology modelling)[72]. Electron density mapping and quaternary structure visualization allows for exploration of both structural and thermodynamic properties of target receptors and are useful for identifying or revealing drug binding sites and exploring a drug's mode of action. This can be supported by virtual screening searches of compound libraries of interest to identify possible drug candidates based on the protein binding sites or the pharmacophore being developed[73]. A pharmacophore is a group of the minimum structural or chemical characteristic to achieve target binding, based on knowledge of established ligands or a receptor cavity structure and amino acid content. *de novo* virtual drug design can take advantage of the pharmacophore to modify existing chemotypes to design small molecules with expected high affinity and optimised physicochemical properties[73]. Crystal structures for both GPVI and CLEC-2 are also available, both with and without a ligand, providing an opportunity for conducting virtual drug design.

However, since neither GPVI nor CLEC-2 have a high affinity ligand, ligand-based drug design studies are unlikely to provide ligand and so a structure-based virtual study or high through put screen study is more appropriate. Molecular dynamic (MD) simulations can also be implemented when available, especially when receptor flexibility is high or the binding site is known [74]. In the case of GPVI, MD simulations for mapping the binding of the monoclonal antibody 10B12 complex have been reported [75]. Similarly, MD

predictions have been used to model dimeric CLEC-2 and to suggest that its flexibility may contribute to the ligand binding[85]. MD simulations can also reveal protein conformations, allosteric and hidden binding sites[74], which may be easier to target with small molecules.

High throughput screening

HTS assays are widely used for drug development in industry, and more recently in academia. They are performed with tens to hundreds of thousands of compounds, thereby increasing the probability of identifying leads to rationalize further structural design. A typical screening ligand concentration is between 5 to 10 μM in view of the anticipated low affinity, which is 3-4 orders of magnitude above the concentration that is likely to be required for therapeutic application.

A miniaturised Ca^{2+} mobilisation assay is a cell-based assay that is used in most screening platforms. However, it cannot predict whether inhibition is mediated through a protein in a downstream signalling pathway or by blocking the ligand-receptor interaction. Therefore, to identify an inhibitor for protein-protein interactions, a biochemical assay is required to directly monitor the ligand-receptor interaction such as an ELISA or AlphaScreen (Figure 6). The latter is a homogenous method based on the proximity between two species of beads. The protein-protein interaction will bring acceptor and donor beads closer than 200 nm and thereby emit a luminescent type energy[76]. ~~Beyond the selection of the right biochemical assay, there are several questions that need to be overcome including the need to miniaturise the assay to perform a large scale HTS.~~ Nowadays, most screening laboratories have extensive experience on the development and miniaturisation of assays for specific and challenging targets, such as GPVI or CLEC-2.

Overcoming targeting challenges

Structural characterization

One of the reasons for the attrition of GPVI and CLEC-2 small molecule inhibitors is the lack of an established, highly characterized binding site and a potent ligand. In the case of GPVI and CLEC-2, the binding site of their endogenous ligands have been resolved using x-ray crystallography. However, the ligand binding site is that for a protein core with a defined quaternary structure rather than a high affinity single molecule.

An attempt to identify small molecules through a structure-based drug design using NMR and *in silico* modelling was described by Ono *et al.*[77]. The technique takes advantage of the interaction network that a peptide and small molecule develops within a binding site[31], in this case losartan, to map the common moieties that are responsible for receptor binding. However, the binding site cavity resides on the surface of the receptor, rather than in a deep hydrophobic pocket, and the ligand affinity is not optimal for this approach. Nevertheless, most of the *in silico* studies for the development of other small molecule inhibitors of GPVI (Figure 3) use the same binding site[63, 67], with an emphasis on including a hydrogen bond on Lys41 as a screening filter.

The NMR approach of Ono *et al.*[77] relies on real-time ligand exchange that compete within detection limits and is thus dependent on the ligand off-rates. NMR spectroscopy is highly effective in disclosing interactions of low affinity complexes ($K_D < 10^{-6}$ M) and high flexibility ligands[78, 79]. This presents a limitation however when developing high affinity ligands. An alternative approach such as mapping the binding sites of potent proteic inhibitors such as antibodies ~~by crystallography and related techniques~~ and using these to develop small molecule inhibitors are likely to be more successful, albeit ~~more~~ challenging.

Compound library selection

A HTS designed to target GPVI or CLEC-2 should be based on a chemical library or natural compound library oriented to PPI with a wider chemical space than **conventional** drug-like compounds ~~that fulfil Lipinski's rule of five~~[80]. **Conventional small molecules libraries are built on the basis Lipinski's 'rule of five', which predict bioavailability and security of the hit candidates. This limits conventional chemical libraries to compounds with a molecular weight of less than 500 Da and low hydrophobicity (LogP < 5). Therefore, the hit rate of identifying potent small molecule leads of protein-protein interactions by screening libraries is extremely low. Alternatively, small molecules could block GPVI or CLEC-2 through an allosteric action where the requirement for high affinity is less stringent. Potentially, they may also bind to a hotspot segment of the endogenous ligand binding site, as in the case of RGD mimetics and integrin inhibition. Small molecule-based screening and in silico prediction of hotspots[81–83] could suggest areas in GPVI and CLEC-2 to target, although as yet no such areas have been identified.**

The comprehensive analysis of the physicochemical parameters of PPI inhibitors has in most cases identified the need for a larger size and higher hydrophobicity than conventional small molecule drug-like molecules. Thus more complex PPI inhibitor profiles have been used to develop chemical libraries oriented to target PPI, such as the rule of four[84, 85]. In general, most of the inhibitors of PPI are based on derivatives of natural compounds, or in some case, they are macrocycles or products of cyclization of aliphatic chains, due to their larger size and more complex structure. This approach would predict a higher rate of hit identification and would provide a more complex scaffold for further chemical optimization, to increase affinity and bioavailability for oral administration of the lead compounds.

Conclusions

It is becoming clear that in order to compete with multivalent endogenous ligands such as collagen and podoplanin, small molecule ligands should ideally demonstrate a slow off-rate and nanomolar affinity. Therefore, understanding the structure of the binding interface between a ligand-receptor complex is crucial when selecting an appropriate compound library for screening. Compound libraries should include more diverse structures than many of the simple chemical libraries, such as natural compounds or macrocycles. Alternatively, these receptors **could** be targeted by potent proteic inhibitors, such as antibodies, antibody fragments and nanobodies to yield critical information on the binding site to aid development of **high** affinity compounds. Once a high affinity **ligand** has been established, research should then be focused in obtaining as much structural data as possible to get a deeper understanding of the nature of the binding site, including whether inhibition is an orthosteric or an allosteric site. This approach would lead to the establishment of a lead that utilizes the interaction potential and physicochemical properties required for targeting challenging receptor interfaces and offer a viable alternative to traditional screening. Whether this will succeed for GPVI and CLEC-2 remains to be seen, but success will likely be critically dependent on further structures of binding of protein-based ligands such as antibodies and nanobodies.

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References

- [1] Rayes J, Watson SP, Nieswandt B. Functional significance of the platelet immune receptors GPVI and CLEC-2. *J Clin Invest* 2019;129:12-23.
- [2] Nieswandt B, Pleines I, Bender M. Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke. *J Thromb Haemost* 2011;9:92-104.
- [3] Deppermann C. Platelets and vascular integrity. *Platelets* 2018;29:549-555.
- [4] Bigalke B, Haap M, Stellos K, Geisler T, Seizer P, Kremmer E, Overkamp D, Gawaz M. Platelet glycoprotein VI (GPVI) for early identification of acute coronary syndrome in patients with chest pain. *Thromb Res* 2010;125:E184-E189.
- [5] Andrews RK, Arthur JF, Gardiner EE. Targeting GPVI as a novel antithrombotic strategy. *J Blood Med* 2014; 21;5:59-68.
- [6] Suzuki-Inoue K, Fuller GLJ, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood* 2006;107:542-549.
- [7] Finney BA, Schweighoffer E, Navarro-Nunez L, Benezech C, Barone F, Hughes CE, Langan SA, Lowe KL, Pollitt AY, Mourao-Sa D, et al. CLEC-2 and Syk in the megakaryocytic/platelet lineage are essential for development. *Blood* 2012;119:1747-1756.
- [8] Lowe KL, Navarro-Nunez L, Benezech C, Nayar S, Kingston BL, Nieswandt B, Barone F, Watson SP, Buckley CD, Desanti GE. The expression of mouse CLEC-2 on leucocyte subsets varies according to their anatomical location and inflammatory state. *Eur J Immunol* 2015;45:2484-2493.
- [9] Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: A platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood*. 2000;96:1798-1807
- [10] Moroi M, Jung SM, Okuma M, Shinmyozu K. A Patient with Platelets Deficient in Glycoprotein-Vi That Lack Both Collagen-Induced Aggregation and Adhesion. *J Clin Invest* 1989;84:1440-1445.
- [11] Kojima H, Moroi M, Jung SM, Goto S, Tamura N, Kozuma Y, Suzukawa K, Nagasawa T. Characterization of a patient with glycoprotein (GP) VI deficiency

possessing neither anti-GPVI autoantibody nor genetic aberration. *J Thromb Haemost* 2006;4:2433-2442.

[12] Nurden AT, Nurden P. Congenital platelet disorders and understanding of platelet function. *Brit J Haematol* 2014;165:165-178.

[13] Arthur JF, Dunkley S, Andrews RK. Platelet glycoprotein VI-related clinical defects. *Brit J Haematol* 2007;139:363-372.

[14] Hughes CE, Navarro-Núñez L, Finney BA, Mourão-Sá D, Pollitt AY, Watson SP. CLEC-2 is not required for platelet aggregation at arteriolar shear. *J Thromb Haemost* 2010;10:2328-2332.

[15] May F, Hagedorn I, Pleines I, Bender M, Vogtle T, Eble J, Elvers M, Nieswandt B. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood* 2009;114:3464-3472.

[16] Schulte V, Rabie T, Prostredna M, Aktas B, Gruner S, Nieswandt B. Targeting of the collagen-binding site on glycoprotein VI is not essential for in vivo depletion of the receptor. *Blood* 2003;101:3948-3952.

[17] Nieswandt B, Schulte V, Bergmeier W, Mokhtari-Nejad R, Rackebrandt K, Cazenave JP, Ohlmann P, Gachet C, Zirngibl H. Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med* 2001;193:459-469.

[18] Martin EM, Zuidschewoude M, Morán LA, García A, Watson SP. Platelet CLEC-2: dimerisation, clustering and signalling. *Platelets* in press 2021.

[19] Clark JC, Damaskinak F-N, Cheung YFH, Slater A, Watson SP. Structure-function relationship of the platelet glycoprotein VI (GPVI) receptor: does it matter if it is a dimer or monomer? *Platelets* in press 2021.

[20] Li HQ, Lockyer S, Concepcion A, Gong XQ, Takizawa H, Guertin M, Matsumoto Y, Kambayashi J, Tandon NN, Liu YG. The fab fragment of a novel anti-GPVI monoclonal antibody, OM4, reduces in vivo thrombosis without bleeding risk in rats. *Arterioscl Throm Vas* 2007;27:1199-1205.

[21] Nicolson PLR, Nock SH, Hinds J, Garcia-Quintanilla L, Smith CW, Campos J, Brill A, Pike JA, Khan AO, Poulter NS, Kavanagh DM, Watson S, Watson CN, Clifford H, Huissoon AP, Pollitt AY, Eble JA, Pratt G, Watson SP, Hughes CE. Low dose Btk inhibitors selectively block platelet activation by CLEC-2. *Haematologica* 2020. 105:xxx.

[22] Lecut C, Feeney LA, Kingsbury G, Hopkins J, Lanza F, Gachet C, Villeval JL, Jandrot-Perrus M. Human platelet glycoprotein VI function is antagonized by monoclonal anti body-derived Fab fragments. *J Thromb Haemost*. 2003;1:2653-2662.

[23] Gitz E, Pollitt AY, Gitz-Francois JJ, Alshehri O, Mori J, Montague S, Nash GB, Douglas MR, Gardiner EE, Andrews RK, et al. CLEC-2 expression is maintained on activated platelets and on platelet microparticles. *Blood* 2014;124:2262-2270.

- [24] Lebozec K, Jandrot-perrus M, Avenard G, Favre-bulle O, Billiald P, Lebozec K, et al. Design, development and characterization of ACT017 , a humanized Fab that blocks platelet ' s glycoprotein VI function without causing bleeding risks. *MAbs*. 2017;0:1–14.
- [25] Harbi M, Smith C, Nicolson PRN, Watson SP and Thomas MR (2021) Novel antiplatelet strategies targeting GPVI, CLEC-2 and tyrosine kinases Platelets in press
- [26] Sheng CQ, Dong GQ, Miao ZY, Zhang WN, Wang W. State-of-the-art strategies for targeting protein-protein interactions by small-molecule inhibitors - *Chem Soc Rev* 2015;44:8375-8375
- [27] Jones S, Thornton JM. Principles of protein-protein interactions. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(1):13-20.
- [28] Rognan D. Rational design of protein-protein interaction inhibitors. *Medchemcomm* 2015;6:51-60.
- [29] Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? *Blood* 2003;102:449-461.
- [30] Watson SP, Herbert JM, Pollitt AY. GPVI and CLEC-2 in hemostasis and vascular integrity. *J Thromb Haemost* 2010; 8;7:1456-67.
- [31] Kato-Takagaki K, Mizukoshi Y, Yoshizawa Y, Akazawa D, Torii Y, Ono K, Tanimura R, Shimada I, Takahashi H. Structural and Interaction Analysis of Glycoprotein VI-binding Peptide Selected from a Phage Display Library. *J Biol Chem* 2009;284:10720-10727.
- [32] Dutting S, Bender M, Nieswandt B. Platelet GPVI: a target for antithrombotic therapy?! *Trends Pharmacol Sci* 2012;33:583-590.
- [33] Smethurst PA, Onley DJ, Jarvis GE, O'Connor MN, Knight CG, Herr AB, Ouwehand WH, Farndale RW. Structural basis for the platelet-collagen interaction - The smallest motif within collagen that recognizes and activates platelet glycoprotein VI contains two glycine-proline-hydroxyproline triplets. *J Biol Chem* 2007;282:1296-1304.
- [34] Slater A, Perrella G, Onselaer MB, Martin EM, Gauer JS, Xu RG, Heemskerk JWM, Ariens RAS, Watson SP. Does fibrin(ogen) bind to monomeric or dimeric GPVI, or not at all? *Platelets* 2019;30:281-289.
- [35] Kato K, Kanaji T, Russell S, Kunicki TJ, Furihata K, Kanaji S, Marchese P, Reininger A, Ruggeri ZM, Ware J. The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. *Blood* 2003;102:1701-1707.
- [36] Lockyer S, Okuyama K, Begum S, Le S, Sun B, Watanabe T, Matsumoto Y, Yoshitake M, Kambayashi J, Tandon NN. GPVI-deficient mice lack collagen responses and are protected against experimentally induced pulmonary thromboembolism. *Thromb Res* 2006;118:371-380.
- [37] Lecut C, Arocas V, Ulrichs H, Elbaz A, Villeval JL, Lacapere JJ, Deckmyn H, Jandrot-Perrus M. Identification of residues within human glycoprotein VI involved in

the binding to collagen - Evidence for the existence of distinct binding sites. *J Biol Chem* 2004;279:52293-52299.

[38] Bourne JH, Colicchia M, Di Y, Martin E, Slater A, Roumenina LT, Dimitrov JD, Watson SP, Rayes J. Heme induces human and mouse platelet activation through C-type-lectin-like receptor-2. *Haematologica* 2020; 105:xxx.

[39] Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries - Podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* 1999;154:385-394.

[40] Christou CM, Pearce AC, Watson AA, Mistry AR, Pollitt AY, Fenton-May AE, Johnson LA, Jackson DG, Watson SP, O'Callaghan CA. Renal cells activate the platelet receptor CLEC-2 through podoplanin. *Biochem J* 2008;411:133-140.

[41] Astarita JL, Acton SE, Turley SJ. Podoplanin: emerging functions in development the immune system and cancer. *Front Immunol* 2012;3:283;1-11.

[42] Takagi S, Sato S, Oh-hara T, Takami M, Koike S, Mishima Y, Hatake K, Fujita N. Platelets Promote Tumor Growth and Metastasis via Direct Interaction between Aggrus/Podoplanin and CLEC-2. *Plos One* 2013;8;1-11.

[43] Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, Narimatsu H, Ozaki Y. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem* 2007;282:25993-26001.

[44] Nagae M, Morita-Matsumoto K, Kato M, Kaneko MK, Kato Y, Yamaguchi Y. A Platform of C-type Lectin-like Receptor CLEC-2 for Binding O-Glycosylated Podoplanin and Nonglycosylated Rhodocytin. *Structure* 2014;22:1711-1721.

[45] Takemoto A, Miyata K, Fujita N. Platelet-activating factor podoplanin: from discovery to drug development. *Cancer Metast Rev* 2017;36:225-234.

[46] Lonsdale R, Ward RA. Structure-based design of targeted covalent inhibitors. *Chem Soc Rev.* 2018;47:3816–30.

[47] Ran X, Gestwicki JE. Inhibitors of protein–protein interactions (PPIs): an analysis of scaffold choices and buried surface area. *Curr Opin Chem Biol.* 2018;44:75–86.

[48] Podolnikova NP, Yakovlev S, Yakubenko VP, Wang X, Gorkun OV, Ugarova TP. The Interaction of Integrin α (IIb) β (3) with Fibrin Occurs through Multiple Binding Sites in the α (IIb) β -Propeller Domain. *J Biol Chem* 2014;289:2371-2383.

[49] Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. *J Cell Sci* 2006;119:3901-3903.

[50] Xiao T, Takagi J, Collier BS, Wang JH, Springer TA. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* 2004;432:59-67.

- [51] Andronati SA, Karaseva TL, Krysko AA. Peptidomimetics - Antagonists of the fibrinogen receptors: Molecular design, structures, properties and therapeutic applications. *Curr Med Chem* 2004;11:1183-1211.
- [52] Cook JJ, Bednar B, Lynch JJ, Gould RJ, Egbertson MS, Halczenko W, Duggan ME, Hartman GD, Lo MW, Murphy GM, et al. Tirofiban (Aggrastat (R)). *Cardiovasc Drug Rev* 1999;17:199-224.
- [53] Puri A, Bansal A, Sethi R, Dwivedi SK, Narain V, Saran RK, Puri VK. Comparative Assessment of Platelet Glycoprotein (IIb/IIIa) Receptor Occupancy Ratio with Eptifibatide and Tirofiban. *Am J Cardiol* 2009;104:170d-170d.
- [54] Liu FQ, Craft RM, Morris SA, Carroll RC. Lotrafiban: an oral platelet glycoprotein IIb/IIIa blocker. *Expert Opin Inv Drug* 2000;9:2673-2687.
- [55] Harrington RA, Armstrong PW, Graffagnino C, Van de Werf F, Kereiakes DJ, Sigmon KN, Card T, Joseph DM, Samuels R, Granett J, et al. Dose-finding, safety, and tolerability study of an oral platelet glycoprotein IIb/IIIa inhibitor, lotrafiban, in patients with coronary or cerebral atherosclerotic disease. *Circulation* 2000;102:728-735.
- [56] Smethurst PA, Joutsu-Korhonen L, O'Connor MN, Wilson E, Jennings NS, Garner SF, Zhang YJ, Knight CG, Dafforn TR, Buckle A, et al. Identification of the primary collagen-binding surface on human glycoprotein VI by site-directed mutagenesis and by a blocking phage antibody. *Blood* 2004;103:903-911.
- [57] O'Connor MN, Smethurst PA, Farndale RW, Ouwehand WH. Gain- and loss-of-function mutants confirm the importance of apical residues to the primary interaction of human glycoprotein VI with collagen. *J Thromb Haemost* 2006;4:869-873.
- [58] Brondijk THC, de Ruiter T, Ballering J, Wienk H, Lebbink RJ, van Ingen H, Boelens R, Farndale RW, Meyaard L, Huizinga EG. Crystal structure and collagen-binding site of immune inhibitory receptor LAIR-1: unexpected implications for collagen binding by platelet receptor GPVI. *Blood* 2010;115:1364-1373.
- [59] Horii K, Kahn ML, Herr AB. Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. *Blood*. 2006;108:936-42.
- [60] Watson AA, Eble JA, O'Callaghan CA. Crystal structure of rhodocytin, a ligand for the platelet-activating receptor CLEC-2. *Protein Sci* 2008;17:1611-1616.
- [61] Sekiguchi T, Takemoto A, Takagi S, Takatori K, Sato S, Takami M, Fujita N. Targeting a novel domain in podoplanin for inhibiting platelet-mediated tumor metastasis. *Oncotarget* 2016;7:3934-3946.
- [62] Watson AA, Brown J, Harlos K, Eble JA, Walter TS, O'Callaghan CA. The crystal structure and mutational binding analysis of the extracellular domain of the platelet-activating receptor CLEC-2. *J Biol Chem* 2007;282:3165-3172.
- [63] Taylor L, Vasudevan SR, Jones CI, Gibbins JM, Churchill GC, Campbell RD, Coxon CH. Discovery of Novel GPVI Receptor Antagonists by Structure-Based Repurposing. *Plos One* 2014;9;6:1-10.

- [64] Lee TY, Chang CC, Lu WJ, Yen TL, Lin KH, Geraldine P, Li JY, Sheu JR. Honokiol as a specific collagen receptor glycoprotein VI antagonist on human platelets: Functional ex vivo and in vivo studies. *Sci Rep* 2017;7:1-13.
- [65] Lin KH, Kuo JR, Lu WJ, Chung CL, Chou DS, Huang SY, Lee HC, Sheu JR. Hinokitiol inhibits platelet activation ex vivo and thrombus formation in vivo. *Biochem Pharmacol* 2013;85:1478-1485.
- [66] Chen TG, Lee JJ, Lin KH, Shen CH, Chou DS, Sheu JR. Antiplatelet activity of caffeic acid phenethyl ester is mediated through a cyclic GMP-dependent pathway in human platelets. *Chinese J Physiol* 2007;50:121-126.
- [67] Bhunia SS, Misra A, Khan IA, Gaur S, Jain M, Singh S, Saxena A, Hohlfield T, Dikshit M, Saxena AK. Novel Glycoprotein VI Antagonists as Antithrombotics: Synthesis, Biological Evaluation, and Molecular Modeling Studies on 2,3-Disubstituted Tetrahydropyrido(3,4-b)indoles. *J Med Chem* 2017;60:322-337.
- [68] Misra A, Prakash P, Aggarwal H, Dhankani P, Kumar S, Pandey CP, Pugh N, Bihan D, Barthwal MK, Farndale RW, et al. Anti-thrombotic efficacy of S007-867: Pre-clinical evaluation in experimental models of thrombosis in vivo and in vitro. *Biochem Pharmacol* 2018;148:288-297.
- [69] Onselaer MB, Nagy M, Pallini C, et al. Comparison of the GPVI inhibitors losartan and honokiol. *Platelets*. 2020;31(2):187-197.
- [70] Tsukiji N, Osada M, Sasaki T, Shirai T, Satoh K, Inoue O, Umetani N, Mochizuki C, Saito T, Kojima S, et al. Cobalt hematoporphyrin inhibits CLEC-2-podoplanin interaction, tumor metastasis, and arterial/venous thrombosis in mice. *Blood Adv* 2018;2:2214-2225.
- [71] Chang YW, Hsieh PW, Chang YT, Lu MH, Huang TF, Chong KY, Liao HR, Cheng JC, Tseng CP. Identification of a novel platelet antagonist that binds to CLEC-2 and suppresses podoplanin-induced platelet aggregation and cancer metastasis. *Oncotarget*. 2015;6:42733-42748.
- [72] Thompson MC, Yeates TO, Rodriguez JA. Advances in methods for atomic resolution macromolecular structure determination. *F1000Research*. 2020;9:1-18.
- [73] Lin XQ, Li X, Lin XB. A Review on Applications of Computational Methods in Drug Screening and Design. *Molecules* 2020;25.1375; 1-12.
- [74] Choi SB, Yap BK, Choong YS, Wahab H. Molecular dynamics simulations in drug discovery. *Encycl Bioinforma Comput Biol ABC Bioinforma*. 2018;1-3:652-65.
- [75] Liu W, Liu G, Zhou H, Fang X, Fang Y, Wu J. Computer prediction of paratope on antithrombotic antibody 10B12 and epitope on platelet glycoprotein VI via molecular dynamics simulation. *Biomed Eng Online*. 2016;15(Suppl 2):152;648-658.
- [76] Ullman EF, Kirakossian H, Singh S, et al. Luminescent oxygen channeling immunoassay: measurement of particle binding kinetics by chemiluminescence. *Proc Natl Acad Sci U S A*. 1994;91(12):5426-5430.

- [77] Ono K, Takeuchi K, Ueda H, Morita Y, Tanimura R, Shimada I, Takahashi H. Structure-Based Approach To Improve a Small-Molecule Inhibitor by the Use of a Competitive Peptide Ligand. *Angew Chem Int Edit* 2014;53:2597-2601.
- [78] Ziarek JJ, Peterson FC, Lytle BL, Volkman BF. Binding Site Identification and [78] Structure Determination of Protein-Ligand Complexes by Nmr: A Semiautomated Approach. *Method Enzymol* 2011;493:241-275.
- [79] Fernandez-Alonso MD, Diaz D, Berbis MA, Marcelo F, Canada J, Jimenez-Barbero J. Protein-Carbohydrate Interactions Studied by NMR: From Molecular Recognition to Drug Design. *Curr Protein Pept Sc* 2012;13:816-830.
- [80] Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliver Rev* 1997;23:3-25.
- [81] Qiao Y, Xiong Y, Gao H, Zhu X, Chen P. Protein-protein interface hot spots prediction based on a hybrid feature selection strategy. *BMC Bioinformatics* 2018;19;14:1-16.
- [82] Wang H, Liu C, Deng L. Enhanced Prediction of Hot Spots at Protein-Protein Interfaces Using Extreme Gradient Boosting. *Sci Rep.* 2018; 8:14285;1-13.
- [83] Ibarra AA, Bartlett GJ, Hegedus Z, Dutt S, Hobor F, Horner KA, Hetherington K, Spence K, Nelson A, Edwards TA, et al. Predicting and Experimentally Validating Hot-Spot Residues at Protein-Protein Interfaces. *Acs Chem Biol* 2019;14:2252-2263.
- [84] Hamon V, Bourgeas R, Ducrot P, Theret I, Xuereb L, Basse MJ, Brunel JM, Combes S, Morelli X, Roche P. 2P2I(HUNTER): a tool for filtering orthosteric protein-protein interaction modulators via a dedicated support vector machine. *J R Soc Interface* 2014;11;1-11.
- [85] Hamon V, Brunel JM, Combes S, Basse MJ, Roche P, Morelli X. 2P2Ichem: focused chemical libraries dedicated to orthosteric modulation of protein-protein interactions. *Medchemcomm* 2013;4:797-809.
- [86] Jiang P, Loyau S, Tchitchinadze M, Ropers J. Inhibition of Glycoprotein VI Clustering by Collagen as a Mechanism of Inhibiting Collagen-Induced Platelet Responses: The Example of Losartan. *PLoSOne* 2015;10(6):1–20.