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Affimer reagents as tool molecules to modulate platelet GPVI-ligand interactions and specifically bind GPVI dimer

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Abstract:

Glycoprotein (GP)VI plays a key role in collagen-induced platelet aggregation. Affimers are engineered binding protein alternatives to antibodies. We screened and characterized GPVI-binding Affimers as novel tools to probe GPVI function. Among the positive clones, M17, D22 and D18 bound GPVI with the highest affinities (KD in the nM range). These Affimers inhibited GPVI-CRP-XL/collagen interactions, CRP-XL/collagen induced platelet aggregation and D22 also inhibited in vitro thrombus formation on a collagen surface under flow. D18 bound GPVI dimer but not monomer. GPVI binding was increased for D18 but not M17/D22 upon platelet activation by CRP-XL and ADP. D22 but not M17/D18 displaced nanobody2 (Nb2) binding to GPVI, indicating similar epitopes for D22 with Nb2 but not for M17/D18. Mapping of binding sites revealed that D22 binds a site that overlaps with Nb2 on the D1-domain, while M17 targets a site on the D2-domain, overlapping in part with the glenzocimab binding site, a humanized GPVI antibody Fab-fragment. D18 targets a new region on the D2-domain. We found that D18 is a stable non-covalent dimer and forms a stable complex with dimeric GPVI with 1:1 stoichiometry. Taken together, our data demonstrate that Affimers modulate GPVI-ligand interactions and bind different sites on GPVI D1/D2-domains. D18 is dimer-specific and could be used as a tool to detect GPVI dimerization or clustering in platelets. A dimeric epitope regulating ligand binding was identified on the GPVI D2-domain, which could be used for the development of novel bivalent antithrombotic agents selectively targeting GPVI dimer on platelets.

Conflict of interest: COI declared - see note

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1 **Affimer reagents as tool molecules to modulate platelet GPVI-ligand interactions and**
2 **specifically bind GPVI dimer**

3
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5
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32 For original data, please contact r.xu1@leeds.ac.uk

33 Raw HDX-MS data are available at ProteomeXchange Consortium via the PRIDE partner
34 repository under the dataset identifier PXD046982.

37 **KEY POINTS**

- 38 • We generated Affimers against platelet GPVI and mapped their binding sites, revealing
39 functional regions regulating ligand binding.
- 40 • A dimeric epitope was identified on GPVI for Affimer D18 which specifically binds GPVI
41 dimer through a 1:1 interaction.

42

43 **ABSTRACT**

44 Glycoprotein (GP)VI plays a key role in collagen-induced platelet aggregation. Affimers are
45 engineered binding protein alternatives to antibodies. We screened and characterized GPVI-
46 binding Affimers as novel tools to probe GPVI function. Among the positive clones, M17,
47 D22 and D18 bound GPVI with the highest affinities (K_D in the nM range). These Affimers
48 inhibited GPVI-CRP-XL/collagen interactions, CRP-XL/collagen induced platelet aggregation
49 and D22 also inhibited *in vitro* thrombus formation on a collagen surface under flow. D18
50 bound GPVI dimer but not monomer. GPVI binding was increased for D18 but not M17/D22
51 upon platelet activation by CRP-XL and ADP. D22 but not M17/D18 displaced nanobody2
52 (Nb2) binding to GPVI, indicating similar epitopes for D22 with Nb2 but not for M17/D18.
53 Mapping of binding sites revealed that D22 binds a site that overlaps with Nb2 on the D1-
54 domain, while M17 targets a site on the D2-domain, overlapping in part with the glenzocimab
55 binding site, a humanized GPVI antibody Fab-fragment. D18 targets a new region on the D2-
56 domain. We found that D18 is a stable non-covalent dimer and forms a stable complex with
57 dimeric GPVI with 1:1 stoichiometry. Taken together, our data demonstrate that Affimers
58 modulate GPVI-ligand interactions and bind different sites on GPVI D1/D2-domains. D18 is
59 dimer-specific and could be used as a tool to detect GPVI dimerization or clustering in
60 platelets. A dimeric epitope regulating ligand binding was identified on the GPVI D2-domain,
61 which could be used for the development of novel bivalent antithrombotic agents selectively
62 targeting GPVI dimer on platelets.

63

64 INTRODUCTION

65

66 Glycoprotein VI (GPVI) is a platelet receptor that plays important roles in haemostasis and
67 pathological processes such as arterial and venous thrombosis¹. Upon vascular trauma or
68 atherosclerotic plaque rupture, GPVI interacts with the exposed sub-endothelial collagen,
69 initiating a signalling cascade for platelet activation and blood clot formation² (supplemental
70 Figure 1). Recent studies have indicated that GPVI also supports thrombus growth via its
71 interaction with fibrin³⁻⁵. The GPVI extracellular region is composed of immunoglobulin-like
72 domains D1 and D2. The collagen binding site is localised in D1⁶ (supplemental Figure 2A).
73 Some studies have indicated a role for D2 in receptor dimerization^{7,8}. GPVI is expressed
74 either as a mixture of monomers and dimers, or predominantly monomers on resting
75 platelets, with the binding of ligands, e.g. collagen and fibrin, inducing higher-order clustering
76 and platelet signalling⁹⁻¹¹. Dimerization of GPVI on platelets is stabilized through an intra-
77 molecular disulphide bond in the cytoplasmic region¹². The number of GPVI dimers has been
78 reported to increase upon platelet activation^{9,10}. Crystallographic studies have shown that
79 GPVI can be either monomeric or dimeric^{6-8,13,14}. Despite data suggesting the existence of
80 dimeric GPVI, GPVI extracellular domains associate with each other weakly and no clear
81 dimer formation has been observed in solution^{7,15}.

82

83 GPVI could be a promising drug target for novel anti-thrombotic molecules with a low
84 bleeding risk^{16,17}. Understanding functional sites on GPVI that regulate GPVI-ligand
85 interactions provides valuable information to help guide inhibitor design. Recently, two
86 functional sites were identified on GPVI by structural studies using nanobodies (Nb) and a
87 Fab-fragment. The first is on the D1-domain, adjacent to the Collagen Related Peptide
88 (CRP) binding site. Binding of Nb2 to this site allosterically inhibited collagen/CRP binding
89 and platelet aggregation^{8,14} (supplemental Figure 2A). In the Nb2 bound crystal structure,
90 GPVI adopts a D2-domain-swapped dimer conformation (supplemental Figure 2B). The
91 domain-swap is mediated by the C-C' hinge loop, possibly playing an important role in
92 platelet signalling. The second functional site occupies a discontinuous region in the D2-
93 domain and includes the C-C' hinge loop region. This site is targeted by glenzocimab, a
94 humanized GPVI antibody Fab-fragment that is under development at the clinical stage¹³. It
95 has been suggested that inhibition by glenzocimab blocks collagen binding through a
96 combination of steric hindrance and allosteric changes. Inhibition of this site also affects
97 GPVI dimerization and clustering¹³.

98

99 Affimers are engineered conformational binding proteins that possess many desirable
100 properties of antibodies, including high specificity and high affinity binding, while additionally

101 featuring substantial stability, simplicity, versatility and cost-effective production^{18,19}. There
102 are two types of Affimers, one based on the human stefin A protein and the second on a
103 consensus plant cystatin sequence^{19,20}. Affimers present 2 variable regions of 9 residues for
104 molecular recognition (supplemental Figure 3). Structurally, Affimers have an alpha-helix
105 positioned above an anti-parallel beta-sheet, which is different than the immunoglobulin fold
106 in antibodies. Compared to antibodies, Affimers are more stable and easily modified to the
107 needs of studying target protein function including the capability of intra-cellular
108 expression²¹. Affimers are isolated from a library comprising $\sim 10^6$ sequences using phage
109 display, which overcomes the costs/ethics associated with animals used for antibody
110 production^{22,23}.

111

112 Here, we screened for Affimers targeting GPVI, and characterized their effect on ligand
113 interactions, platelet aggregation and *in vitro* thrombus formation. We also mapped their
114 binding sites on GPVI and compared binding to GPVI monomer versus dimer. Our data
115 show that Affimers modulate GPVI function and bind different sites on GPVI. GPVI dimer
116 can be specifically targeted by Affimer D18, thus representing a promising novel tool to
117 further understand GPVI dimerization or clustering on platelets. A novel dimeric epitope is
118 identified on GPVI, representing a promising functional site for developing inhibitors
119 selectively targeting GPVI dimer in platelets.

120

121 **Methods**

122

123 The main experimental methods used in this study include ELISA, microscale
124 thermophoresis (MST), platelet aggregation assays, flow cytometry, *in vitro* thrombus
125 formation assays and native/hydrogen deuterium exchange mass spectrometry, which are
126 briefly described below. For further details, please see the supplement. Other methods,
127 including Affimer/GPVI expression and purification, surface plasmon resonance (SPR), pull-
128 down assays, competition ELISA, biontinlyation/fluorescent labelling of Affimer/GPVI,
129 molecular modelling, platelet isolation and blood collection, are described in the supplement.

130

131 ELISA was performed by incubating Affimers with immobilised GPVI-Fc on Maxisorp Nunc-
132 immuno 96-well plates. Bound Affimers were detected using HRP conjugated rabbit anti-6-
133 histag antibody (Cambridge bioscience, Cambridge, UK). Data collection and analysis were
134 performed as previously described¹⁵.

135

136 Microscale thermophoresis was carried out on a NT.115 (NanoTemper GmbH, Munich,
137 Germany) instrument. Alexa Fluor 488 C5 Maleimide labelled Affimers were mixed with
138 increasing concentrations of GPVI proteins. Data collection and analysis were performed as
139 previously described¹⁵.

140

141 For platelet aggregation assays, washed platelets were incubated with Affimers at different
142 concentrations for 15min at 37°C. Aggregation was induced by CRP-XL or collagen and
143 monitored using a Helena AggRAM (Helena Biosciences Europe, Tyne and Wear, UK).

144

145 For flow cytometry, washed platelets were incubated with Affimers conjugated to Alexa
146 Fluor-488, CD42b-APC, ±CRP-XL or ±ADP for 20min, followed by addition of 1%
147 paraformaldehyde/PBS (v/v) to halt the reaction.

148

149 *In vitro* thrombus formation assays were performed using Vena8 biochips (Cellix; Dublin,
150 Ireland). Citrated human whole blood was incubated with DIOC6 for 10min and perfused
151 through collagen coated microfluidic chips at 1000s⁻¹ for 2min ±GPVI Affimers. After flow,
152 non-adherent platelets were washed off with PBS for 3min.

153

154 For native mass spectrometry, mixtures of GPVI proteins and Affimers were buffer
155 exchanged into 0.2M ammonium acetate (pH6.9) before analysis.

156

157 Hydrogen deuterium exchange mass spectrometry was carried out using a liquid handling
158 system (LEAP Technologies) coupled with an Acquity M-Class LC/HDX manager (Waters).
159 Samples were prepared by mixing GPVI proteins and Affimers in 10mM potassium
160 phosphate (pH7.6). The HDX reactions were initialised by deuterated buffer and incubated at
161 4°C for 0.5-10min. The reactions were then quenched, followed by proteolysis and peptide
162 analysis.

163

164 Informed written consent was obtained for blood donations, according to the declaration of
165 Helsinki. Ethical approval was obtained from the School of Medicine Research Ethics
166 Committee (MREC 19-006, University of Leeds).

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RESULTS

Screening of GPVI-targeting Affimers

Two phage-display screens against biotinylated recombinant GPVI monomer and dimer (supplemental Figure 4) were performed. Purified Fc-domain was screened in parallel to eliminate binders to this domain. After three rounds of panning, 2x24 colonies were tested for binding GPVI monomer and dimer by phage ELISA (Figure 1A,B). Sequencing revealed 17 unique binders from the GPVI monomer screen and 14 from the dimer screen. None of the binders interacted with the Fc-domain (Figure 1A,B). In contrast to other Affimers that bound both monomer and dimer, Affimer D18 selectively bound GPVI dimer but not monomer. The Affimers were sub-cloned and expressed in *E. coli* and purified (supplemental Figure 5). Pull-down experiments were used to confirm Affimer interaction with GPVI. We selected 3 Affimers for further study based on pull-down experiments (data not shown) and binding affinity. The affinities of Affimers for GPVI were investigated by titrating Affimers over immobilized GPVI-Fc dimer using ELISA. Affimers, M17, D22 and D18 showed the highest affinities to GPVI dimer with $K_D=3.6\pm0.2$, $13.0\pm1.2\text{nM}$ and 0.14 ± 0.02 respectively (supplemental Figure 6).

Effects of Affimers on GPVI-ligand interactions

Effects of Affimers on interactions of GPVI with collagen and CRP-XL were tested using competitive ELISAs. The Affimers inhibited GPVI interactions with CRP-XL and collagen to varying degrees (Figure 1C,D; supplemental Table 1). Among the 3 Affimers, D22 showed strongest inhibition of GPVI interaction with both ligands, at 98% (CRP-XL) and 84% (collagen). Slightly weaker inhibition of GPVI interactions with CRP-XL and collagen was observed for M17 and D18 (57% and 55% for CRP-XL; 74% and 73% for collagen, respectively). These data show that Affimers modulate GPVI-ligand interactions.

Affimers modulate platelet aggregation

The effects of Affimers on platelet aggregation were characterized using light transmission aggregometry. CRP-XL and collagen were used to induce aggregation with or without pre-incubation with Affimers. Three agonist concentrations were tested to determine the optimal trigger ($10\mu\text{g/mL}$ for CRP-XL and $5\mu\text{g/mL}$ for collagen) for aggregation (supplemental Figure 7). It has been reported that Fab-fragments can trigger GPVI activation and platelet aggregation on their own²⁴. To test the potential direct effect on platelet aggregation,

203 Affimers were added to washed platelets and the percentage of platelet aggregation without
204 additional trigger was recorded. Negligible aggregation (<20%) was observed for M17, D22
205 and D18 (supplemental Figure 8). We next investigated the effect of Affimers on CRP-XL
206 and collagen-mediated aggregation and found that Affimers M17 and D22 inhibited
207 aggregation induced by both agonists (Figure 1E,F; Table 1; supplemental table 1). Inhibition
208 with D18 was only observed in CRP-XL but not collagen induced aggregation at the tested
209 Affimer concentrations. Stronger inhibition of platelet aggregation was observed for all
210 Affimers when CRP-XL was used as agonist compared to collagen. The weaker inhibition
211 when using collagen as agonist is likely due to the presence of receptor $\alpha 2\beta 1$, which has
212 been reported to play a regulatory role in platelet activation by facilitating platelet-collagen
213 but not CRP-XL adhesion^{25,26}. Similar observations have been observed for nanobody2,
214 where a much higher nanobody concentration was required to inhibit collagen but not CRP-
215 XL induced platelet aggregation⁸. To confirm that Affimers are specific for GPVI, we found
216 no effect of the Affimers on thrombin-induced platelet aggregation (supplemental figure 9).

217

218 **Affimers inhibit CRP-XL-GPVI binding with different efficacies**

219 In our competition ELISA, D22 could almost fully inhibit CRP-XL binding to GPVI dimer,
220 whereas M17 and D18 only partially inhibited the interaction (Figure 1C). The distinct
221 inhibition effects for these Affimers suggest different inhibition mechanisms on CRP-XL
222 binding. To further investigate this, we tested Affimers at multiple concentrations by
223 competitive ELISA and determined their maximum inhibition efficacies. Consistent with our
224 previous observations using a single Affimer concentration, we observed that while D22 had
225 a maximum inhibition efficacy of 99%, M17 and D18 only partially inhibited the binding, with
226 maximum efficacies of 69% and 61%, respectively (supplemental Figure 10).

227

228 **Affimer D18 binds GPVI dimer but not monomer**

229 Affimer D18 selectively bound GPVI dimer but not monomer in phage ELISA (clone 18,
230 Figure 1B). To further investigate the selectivity of this Affimer, we developed an ELISA to
231 analyse binding of D18 to immobilized GPVI monomer, dimer and Fc-domain. M17 and D22,
232 which interacted with both GPVI monomer and dimer in the phage ELISA, were also tested.
233 Consistent with the phage ELISA, M17 and D22 bound both monomer and dimer
234 (supplemental Figure 11A, B), while D18 bound GPVI dimer but not monomer (supplemental
235 Figure 11C). No binding was observed for the Affimer scaffold with either monomer or dimer
236 of GPVI, and no GPVI-specific Affimer bound to the Fc-domain (supplemental Figure 11A-
237 C). The K_D of M17 binding to GPVI monomer and dimer was 11 ± 1 nM and 3.6 ± 0.2 nM,
238 respectively (Figure 2A; Table 1; supplemental table 2). D22 binds GPVI dimer at K_D of 13 ± 1
239 nM while binding is not saturable with GPVI monomer and a $K_D > 100$ nM (Affimer

240 concentration that generates half of the maximum binding signal) was estimated from the
241 data (Figure 2B; Table 1; supplemental table 2). The K_D of D18 binding to GPVI dimer was
242 $0.14 \pm 0.02 \text{ nM}$, and no binding of D18 to GPVI monomer was observed at any Affimer
243 concentration (Figure 2C; Table 1; supplemental table 2). Affimer scaffold did not bind GPVI
244 monomer or dimer (Figure 2A-C).

245
246 We next investigated the kinetics of the interactions of D18 with GPVI by SPR. GPVI
247 monomer, dimer and Fc-domain were flowed over immobilised biotinylated D18 on a
248 streptavidin chip, in comparison with M17 and D22. The K_D of M17 to GPVI monomer and
249 dimer was $55 \pm 18 \text{ nM}$ and $4.4 \pm 4.3 \text{ nM}$, respectively (supplemental Figure 12A,B; Table 1;
250 supplemental Table 2 and 3). The K_D of D22 to GPVI monomer and dimer was $53 \pm 11 \text{ nM}$
251 and $5.3 \pm 2.5 \text{ nM}$, respectively (supplemental Figure 12C,D; Table 1; supplemental Table 2
252 and 3). The K_D of D18 to GPVI dimer was $0.23 \pm 0.01 \text{ nM}$, whilst no binding was detected for
253 D18 to GPVI monomer (supplemental Figure 12E,F; Table 1; supplemental Table 2 and 3).
254 No binding was observed for the Fc-domain (supplemental Figure 13).

255
256 Then we used microscale thermophoresis (MST) to study whether selective binding of
257 Affimer D18 to GPVI dimer is also observed in solution. M17 bound GPVI monomer and
258 dimer in MST with $K_D = 105 \pm 31 \text{ nM}$ and $4 \pm 2 \text{ nM}$, respectively (Figure 2D; supplemental Table
259 2). D22 bound GPVI dimer in MST with $K_D = 172 \pm 31 \text{ nM}$. Binding did not reach to saturation
260 with GPVI monomer. The K_D was estimated at $>1 \mu\text{M}$ (GPVI monomer concentration that
261 generates half of the maximum binding signal) based on the data (Figure 2E; supplemental
262 Table 2). D18 bound GPVI dimer at $K_D = 0.5 \pm 0.2 \text{ nM}$, whereas no D18 binding to GPVI
263 monomer was observed (Figure 2F; supplemental Table 2). Taken together, the ELISA, SPR
264 and MST data show that D18 binds GPVI dimer selectively over monomer and that this
265 occurs both in surface- and solution-based reactions.

266
267 Flow cytometry binding assays were then performed to study the (selective) binding of D18
268 to GPVI dimer on platelets. Washed platelets were activated by CRP-XL and ADP in the
269 presence of Alexa-Fluor 488 labelled D18. Labelled M17, D22 and scaffold were also tested
270 in parallel with D18. A significant increase of fluorescence was observed upon CRP-XL and
271 ADP activation of the platelets for D18 binding compared to the scaffold, M17 and D22
272 (Figure 2G,H, supplemental Figure 14). These data indicate that D18 can bind GPVI dimers
273 generated through GPVI clustering upon platelet activation with CRP-XL and ADP.

274

275 **Affimers inhibit thrombus formation under flow**

276 We next investigated the effects of Affimers on *in vitro* thrombus formation under flow
277 conditions by flowing whole blood over a collagen-coated surface for 2min, followed by a
278 3min buffer wash. The rationale for this set-up is to allow platelet adhesion for 2min as per
279 manufacturer instructions for the Cellix VenaFlux system and to ensure platelets are stably
280 adhered after 2min using a 3min buffer wash^{27,28}. Fluorescent images after 2min of blood
281 flow showed less thrombus formation only in the presence of Affimer D22 compared to buffer
282 or scaffold controls (Figure 3A). Thrombus surface coverage was quantified and compared
283 at all time-points before 2min (Figure 3B-F; Table 1). At 2min, a significant reduction in mean
284 surface coverage was only observed with D22 (1.8%) but not in the presence of M17(3.8%)
285 or D18 (4.1%) compared to buffer or scaffold controls (7.8% and 6.5%, respectively) (Figure
286 3B). These data show that Affimers D22 inhibit thrombus formation under flow.

287

288 **Competition of Affimers with Nb2 for GPVI**

289 Slater *et al.* recently reported that GPVI Nb2 binds GPVI in the D1-domain, supported by a
290 new GPVI-Nb2 crystal structure⁸. To investigate where Affimers bind GPVI in relation to
291 Nb2, we performed competition ELISA by adding Nb2 with Affimers present on immobilized
292 GPVI dimer. At a molar ratio of 20:1 (Affimer:Nb2), we observed strong inhibition by Affimer
293 D22 (90%) on Nb2-GPVI interaction (Figure 4A). These effects were concentration
294 dependent (Figure 4B). No inhibition was found for M17 and D18 (Figure 4A,B). These data
295 indicate that while binding sites of D22 may have some overlap with the Nb2 site, M17 and
296 D18 bind to distinct sites compared to the Nb2 site on GPVI.

297

298 **Affimer binding sites on GPVI**

299 To pinpoint the location where Affimers bind on GPVI, we performed HDX-MS²⁹ on Affimers
300 M17, D22, and the dimer specific Affimer, D18. Several GPVI regions showed strong
301 protection from deuterium exchange upon Affimer binding, including 141Tyr-149Thr on the
302 D2-domain for M17 (Figure 5A; Table 1), and 44Ser-53Leu on the D1-domain for D22
303 (Figure 5B; Table 1). For D18, the protected region included 113Gln-123Phe and, to a lesser
304 extent, 141Tyr-149Thr on the D2-domain (Figure 5C; Table 1). The regions with the
305 strongest protection indicate key interacting sites on GPVI for the Affimers. Binding of M17
306 led to de-protection of 86Val-112Leu on GPVI, indicating allosteric conformational changes
307 upon M17 binding, resulting in destabilization of the hydrogen bonding network and
308 increased flexibility of these regions. Our data suggest that 141Tyr-149Thr is a common site
309 for binding of both M17 and D18. To further investigate the degree of overlap in this region,
310 we performed a competition ELISA of D18 and M17 binding to GPVI. We observed a modest
311 displacement of M17-GPVI interaction by D18 (24%), compared with scaffold (-2%) and D22
312 (-8%) controls, suggesting their binding sites in this region are partially overlapping

313 (supplemental figure 15). Together, the HDX-MS data identified key GPVI binding sites for
314 D22, D18, M17 and an allosteric site upon M17 binding.

315

316 The Affimer binding sites were compared with CRP, Nb2 and glenzocimab sites on GPVI
317 (Figure 5D). We observed that Affimer D22 site overlaps in part with the CRP and Nb2 sites
318 on GPVI D1-domain. Tyr47, involved in both Nb2 and CRP binding, also forms part of the
319 D22 binding site on GPVI D1-domain (supplemental Figure. 2A). The binding site of Affimer
320 M17, 141Tyr-149Thr, includes part of the glenzocimab site on GPVI D2-domain (144Ala-
321 149Leu). No overlap was found for the major binding site of D18 on GPVI, 113Gln-123Phe,
322 for all three ligands. Thus, while the binding sites for D22 and M17 show some degree of
323 overlap with the sites for Nb2 and glenzocimab on GPVI, respectively, D18 largely interacts
324 with a new region on GPVI which does not overlap with any of these known sites.

325

326 **Affimer D18 is a stable dimer**

327 To further understand how dimer-specific Affimer D18 interacts with GPVI dimer, the
328 stoichiometry of this interaction was determined by native mass spectrometry. We observed
329 that D18 was a stable dimer on its own. M17 which was tested as a control was
330 predominantly monomeric (Figure 6A; supplemental Figure 16). This observation is
331 consistent with the larger predicted molecular weight from a calibrated size exclusion column
332 when compared to its theoretical monomeric weight, and when compared with M17
333 (supplemental Figure 17). We next investigated whether D18 forms stable complexes with
334 GPVI monomer and dimer. To minimise the interference of glycosylation in molecular weight
335 determination, a variant of GPVI (N72Q) that does not undergo glycosylation were used in
336 the experiment⁸. We found that no complex formation was observed in a 1:1 molar ratio
337 solution of D18 with GPVI (Figure 6B,C). Stable complex formation was however detected
338 when D18 was added to GPVI-Fc dimer using the same experimental conditions. When
339 GPVI dimer was added to D18 at 1:1 molar ratio, complex formation was observed. The
340 molecular weight of the complex corresponds to 1 GPVI dimer interacting with 1 D18 dimer
341 (1:1 complex). (Figure 6D,E). These data confirm that D18 is a stable dimer and specifically
342 binds GPVI dimer but not monomer.

343

344 **Modelling of GPVI-Affimer interactions**

345 To further analyse how Affimers interact with GPVI at the molecular level, we modelled
346 Affimers M17, D22 and D18 and their interactions with GPVI using HADDOCK³⁰. Residues
347 in the variable loops of Affimers, and those in GPVI identified by HDX-MS, were used as
348 active residues in the docking. For all models generated, the best scoring structure from the
349 top cluster, with the lowest energy was selected. The molecular model for Affimers used in

350 the docking were generated by AlphaFold³¹ (Figure 6F). When the M17-GPVI model (Figure
351 6G) was superposed with glenzocimab-GPVI complex, we observed that the two variable
352 loops of M17 were between the CDR loops of the heavy and light chains of glenzocimab
353 (supplemental Figure 18A). To investigate the possible mechanisms for M17 inhibition on
354 CRP-GPVI binding, we modelled CRP-XL to the M17-GPVI model by structure superposition
355 with the CRP bound GPVI structure. We observed that unlike that reported for glenzocimab,
356 bound CRP did not cause steric hindrance of M17 binding (supplemental Figure 18B,C).
357 Superposition of the D22-GPVI model (Figure 6H) with the Nb2-GPVI complex showed that
358 D22 occupies the same location as Nb2. The two variable loops of D22 were in close
359 proximity with the CDRs of Nb2 (supplemental Figure 18D). Prior to the generation of a D18-
360 GPVI model, using HDX-MS we confirmed that residues in the variable loop region of
361 Affimer D18 are involved in GPVI dimer binding (supplemental Figure 19). In the D18-GPVI
362 model (Figure 6I; supplemental Figure 20), dimeric D18 interacts with the D2-domains from
363 2 symmetrically arranged GPVI molecules. The major and minor D18 binding regions on
364 GPVI, 113Gln-123Phe and 144Ala-149Leu, respectively, form a large binding surface and
365 are close to the variable loops in D18. Together, these docking data provide structural
366 insights into the molecular arrangements of the GPVI/Affimer complexes (Figure 6F-I;
367 supplemental Figure 20; supplemental Figure 21).

368

369

370

371 DISCUSSION

372

373 This study shows that GPVI-CRP-XL/collagen interaction and GPVI mediated platelet
374 aggregation can be modulated by Affimers. Effects of Affimers on the binding of GPVI to
375 fibrin were not probed, which is a limitation of our study. Affimer D22 reduced thrombus
376 formation in whole blood under *in-vitro* flow conditions. The binding sites for Affimers M17,
377 D22 and D18 on GPVI were characterized and shown to represent several functional hot-
378 spots that could play important roles in regulating GPVI ligand binding. Furthermore, we
379 show that D18 is a stable dimer which binds selectively to GPVI dimer with sub-nanomolar
380 affinity. D18 also selectively interacts with GPVI dimer generated by CRP-XL/ADP activation
381 on platelets. These findings imply that D18 can specifically recognize GPVI dimer and thus
382 serve as a promising tool to selectively detect GPVI dimerization or clustering in platelets.

383

384 The configuration of GPVI on resting platelets has been a topic of debate for many years,
385 reported either as predominantly monomer⁹ or as a mixture of monomer and dimer^{10,32}.
386 Other studies suggest a key role for GPVI clustering in platelet function³³. It has been

387 reported that the expression level of GPVI dimers was increased in patients with stroke and
388 obesity^{34,35}. A higher GPVI-dimer level in patients was associated with higher platelet
389 aggregation and P-selectin exposure in response to GPVI-specific agonists or measured by
390 a dimer specific anti-body as compared with healthy controls^{9,34,35}, suggesting that the GPVI
391 dimer could be a potential biomarker and/or anti-thrombotic target. Antibodies and Fabs that
392 specifically recognise GPVI dimer have been previously reported³⁶⁻³⁸. Nevertheless, the
393 location of these dimer specific sites on GPVI have not been identified. Using the dimer
394 specific Affimer D18, the dimeric epitope on GPVI was revealed for the first time, which
395 could be informative for the design of novel anti-thrombotic agents specifically targeting
396 GPVI dimer.

397

398 Our data show that Affimer D18 is a stable dimer itself. Similar Affimer dimers have been
399 reported previously, including those targeting lysine linked di-Ubiquitins. These Affimers bind
400 di-Ubiquitins at high affinity accompanied by slow off rate whereas a much-reduced binding
401 was observed for mono-Ubiquitins³⁹. Structural and biochemical data suggested that linked
402 di-Ubiquitins are conformationally flexible and can adopt distinct conformations in solution,
403 and that Affimer dimers can select and recognise a suitable dimer conformation from the
404 population of conformations that they can adopt⁴⁰. This is reminiscent of our observations for
405 the D18 dimer which specifically binds GPVI dimer, either linked artificially by Fc-domain or
406 on the platelet surface, at high affinity ($K_D=0.2\pm 0.01\text{nM}$) with slow off rate ($1.3\pm 0.04 \times 10^{-4}\text{s}^{-1}$)
407 while negligible binding is observed for the monomer. Based on the above observations, it is
408 possible that the conformational selection mechanism as suggested for Affimers dimers and
409 linked di-Ubiquitins may also apply for D18 dimer and GPVI dimer interaction.

410

411 Our D18-GPVI dimer model reveals a distinct dimeric arrangement of two GPVIs not
412 observed in previous crystallographic studies. The dimer interface is formed by the $\beta\text{C}'$ -E, A-
413 B/F-G loops and βC regions of the D2-domain (supplemental figure 21C). Each D18 dimer
414 subunit interacts with the identified residues in each GPVI dimer subunit through the variable
415 loop. This is different to that of the back-to-back and domain swapped dimer structures
416 (2GI7, 5OU7, 7NMU), where GPVI either interacts with each other through the C-terminal β
417 strand of the D2 domain (βG) (back-to back)^{6,7} or through the βG of D2-domain and $\beta\text{E-F}$
418 strands of the D1-domain (domain swap)⁸.

419

420 Although the binding site for M17 on GPVI overlaps in part with the glenzocimab site, our
421 structure comparisons show that binding of M17 to GPVI is unlikely to cause potential steric
422 hindrance on CRP binding¹³. The much smaller size of Affimers compared to Fab-fragments

423 (12 vs 50 kDa, respectively) may account for the absence of potential steric hindrance in the
424 inhibition. Moreover, M17 does not interact with the C-C' loop which is the major binding site
425 for glenzocimab. These observations suggest that M17 may use a different mechanism to
426 inhibit CRP-GPVI binding compared to glenzocimab. Furthermore, our HDX-MS data
427 suggest that M17-GPVI binding generates a conformational change in the D1-D2 hinge
428 region. Our competition ELISA data show that M17 inhibits CRP binding with a much lower
429 efficacy than that observed for D22, which fully inhibits the binding. These data imply that
430 M17 is likely an allosteric inhibitor that partially inhibits CRP binding on D1-domain through
431 conformational changes generated when binding to the distal D2-domain. Similar allosteric
432 changes at the D1-D2 hinge region have not been observed for glenzocimab in the crystal
433 structure. Further studies are needed to understand how allosteric changes of GPVI
434 generate a partial inhibitory effect of M17 on ligand binding.

435

436 Our HDX-MS data show that D22 bound GPVI at a similar site as nb2. Interestingly, we did
437 not observe deprotection effect on the C-C' loop, suggesting that D22 binding to GPVI
438 should not induce a conformation change in the C-C' loop. This also suggests that the
439 domain swapped GPVI dimer observed in the crystal structure of the nb2-GPVI complex⁸
440 may not exist for the D22-GPVI complex in solution. It is possible that the formation of
441 domain swapped GPVI dimer with bound nb2 is induced by conditions during the
442 crystallisation process, or by differences between nanobody and Affimer binding to GPVI.
443 Further work is needed to investigate if this domain swapped dimer is physiologically
444 relevant.

445

446 In conclusion, we show that Affimers modulate GPVI interaction with collagen/CRP-XL and
447 inhibit CRP-XL and collagen mediated platelet aggregation by GPVI. We observed that
448 Affimers M17, D22 and D18 bind to different sites on GPVI. Using HDX-MS, the Affimers'
449 binding sites on GPVI revealed several regions that play important roles in regulating ligand
450 binding. D22 inhibited *in vitro* thrombus formation. Moreover, we found that Affimer D18
451 selectively binds GPVI dimer but not monomer in platelets, thus representing a promising
452 tool to further understand the role of the GPVI dimerization and clustering in platelet function.
453 Finally, we show that D18 is a stable dimer which forms a 1:1 complex with GPVI dimer. A
454 dimeric epitope on the D2-domain was found which could be used as a promising site for
455 designing anti-thrombotic agents that specifically bind GPVI dimer.

456

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458

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471 spectrometry equipment.

472

473 **AUTHORSHIP CONTRIBUTIONS**

474

475 RGX performed ELISA, SPR, MST and molecular modelling/docking experiments, produced
476 and characterized Affimers, purified GPVI proteins, wrote and edited the manuscript. CT
477 performed phage display screening. ANC performed HDX/native-MS experiment. LTC, RGX
478 and JSG performed platelet aggregation assays. LA performed Affimer sub-cloning and pull-
479 down assays. JSG expressed GPVI-Fc protein. MSH and DMY performed flow cytometry
480 experiment. BAW performed thrombus formation assays. AS provided Nb2. CD, KMN, DCT
481 and SPW contributed to study design. RASA conceived the study, supervised the work,
482 generated the funding and edited the manuscript. All authors read and approved the
483 manuscript.

484

485 **DISCLOSURE OF CONFLICTS OF INTEREST**

486 SPW and AS have a patent for the anti-GPVI nanobodies (WO2022/136457). All authors
487 declare no other conflict of interest.

488

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593

Affimer	Binding sites on GPVI	K_D GPVI monomer (nM)	K_D GPVI dimer (nM)	Platelet aggregation (agonist: collagen/CRP)	Thrombus formation under flow (collagen surface)
M17	141Y-149T	55±18	4.4±4.3	↓ / ↓	-
D22	44S-53L	53±11	5.3±2.5	↓ / ↓	↓
D18	113Q-123F 141Y-149T	N.D.	0.23±0.01	- / ↓	-

595 **Table 1. Summary of the key properties of GPVI Affimers.**

596 Down arrow represents inhibition effect. Hyphen represents no clear inhibition effect. N.D.:
597 not determinable. K_D values were determined using SPR.

598

599 **Figure 1. Identification of GPVI Affimers and their effect on GPVI ligand interactions**
 600 **and platelet aggregation.** Screening of GPVI-binding Affimers raised against (A) GPVI
 601 monomer and (B) GPVI dimer by phage ELISA. Fc-domain (grey) was tested as a control.
 602 No protein was added in Blank (yellow). A total of 31 unique Affimers that bind to
 603 immobilised GPVI monomer (blue) and GPVI dimer (green) with the highest affinity were
 604 identified from 48 clones. These clones are numbered as M1-M24 and D1-D24 for Affimers
 605 screened against GPVI monomer and dimer, respectively. The effect of Affimers M17, D22
 606 and the dimer specific Affimer (D18) on GPVI dimer interaction with immobilised (C) CRP-
 607 XL, (D) collagen were characterised by competitive ELISA, and expressed as % inhibition as
 608 compared to buffer control. Affimer scaffold was also used as a control. The effect of
 609 Affimers M17, D22 and D18 on (E) CRP-XL, (F) collagen-induced platelet aggregation was
 610 studied by aggregation assays. Data was normalised using the scaffold control (100%
 611 aggregation) as reference. Data are presented as mean±SD; n≥3.

612 **Figure 2. Interaction of Affimer M17, D22 and D18 with GPVI monomer and dimer**
 613 **analysed by ELISA, MST and flow-cytometry. ELISA:** (A) M17 bound GPVI monomer
 614 (blue circles) at $K_D=11\pm 1$ nM, and GPVI dimer (red squares) at $K_D=3.6\pm 0.2$ nM. (B) D22
 615 bound GPVI monomer at $K_D=53\pm 11$ nM, and GPVI dimer at $K_D=5.3\pm 2.5$ nM. (C) No binding
 616 was observed for D18 and GPVI monomer. D18 bound GPVI dimer at $K_D=0.14\pm 0.02$ nM. K_D
 617 values were obtained through fitting data with Hill equation. **MST:** M17 bound (D) GPVI
 618 monomer at $K_D=105\pm 31$ nM and GPVI dimer at $K_D=4\pm 2$ nM. D22 bound (E) GPVI monomer
 619 at $K_D=171\pm 36$ nM and GPVI dimer at $K_D>1000$ nM. (F) D18 bound GPVI dimer at
 620 $K_D=0.5\pm 0.2$ nM. No binding was observed for D18 to GPVI monomer. K_D values were
 621 obtained through fitting data with Hill equation. For ELISA and MST, data and K_D were
 622 presented as mean ±SD; n≥3. **Flow-cytometry:** Binding of AlexaFluor-488 labelled Affimers
 623 scaffold, M17, D22 and D18 to washed platelets was analysed by comparing the mean
 624 fluorescent intensity before and after stimulation (Δ MFI) with (G) CRP-XL and (H) ADP.
 625 D18, but not M17 and D22, bound to activated platelets. Friedman test is used to determine
 626 statistical significance (P-values <0.05). Data were presented as mean±SD, n≥4.

627 **Figure 3. Effect of GPVI Affimers on thrombus formation *in vitro*.** Human whole blood
 628 was incubated in the presence or absence of GPVI Affimers (Scaffold, M17, D22 & D18; 10
 629 $\mu\text{g}/\text{mL}$) for 15 min and perfused through collagen (50 $\mu\text{g}/\text{mL}$) coated microfluidic chips at
 630 1000s^{-1} for 2 min. After 2 min of flow, non-adherent platelets were washed off with PBS for 3
 631 min. Images of stably adherent platelets and thrombi were taken by fluorescence microscopy
 632 and quantified using ImageJ. Data presented as (A) representative images (scale = 20 μm)
 633 and (B) % surface coverage at 2 min (Repeated Measures One-Way ANOVA with Šídák's
 634 multiple comparisons test vs scaffold, *p<0.05). (C-F) % Surface Coverage over time up to 2
 635 min. Data presented as mean±SD, n=5.

636 **Figure 4. Competition of Affimers with nanobody 2 for GPVI dimer binding.** (A)
 637 Displacement of Affimers by Nb2-GPVI dimer binding in competition ELISA. Affimers M17,
 638 D22 and D18 (2 μ M) were incubated with 100 nM Nb2 prior to the addition to immobilised
 639 GPVI dimer. (B) Effect of Affimers at multiple concentrations on Nb2-GPVI dimer binding.
 640 Affimer scaffold was used as control. Data were presented as mean \pm SD; n=3.

641 **Figure 5. The location of Affimer binding sites on GPVI for M17, D22 and D18.** The
 642 residues involved in Affimer binding on GPVI determined by HDX-MS for (A) M17, (B) D22
 643 and (C) D18 are displayed and coloured on the crystal structure of GPVI (grey, PDB code:
 644 2GI7). The residues that had strong and weak protection effect upon Affimer binding are
 645 coloured blue and light blue, respectively. The residues that had strong and weak de-
 646 protection effect upon Affimer binding are coloured red and light red, respectively. Red, blue
 647 and grey bars shown in each graph below the GPVI structure represent different GPVI
 648 peptide fragments generated by proteolysis in the presence and absence of Affimers. The
 649 peptide fragment represented by red and blue bars have positive and negative differences in
 650 deuterium uptake, respectively. The peptide fragments represented by grey bars have no
 651 significant changes in deuterium uptake. (D) Representation and comparison of the binding
 652 site residues of M17, D22 and D18 with CRP-XL (cyan), glenzocimab (green) and Nb2
 653 (grey). The amino acids coloured in blue and red had the strong protection and deprotection
 654 effect, respectively, upon the binding of Affimers.

655 **Figure 6. Characterisation of GPVI-Affimer complexes by native mass spectrometry
 656 and molecular modelling.** The native mass spectra of (A) D18 (dimeric, measured
 657 molecular weight is at 21760 Da, approximately two-fold larger than that calculated based on
 658 the monomeric protein sequence, at 11013 Da), (B) GPVI monomer N72Q (monomeric,
 659 measured molecular weight is at 21380 Da, similar to that based on the monomeric protein
 660 sequence, at 21249 Da), (C) GPVI monomer N72Q with D18 (no complex formation
 661 detected, measured molecular weight is at 21380 and 21810 Da for GPVI and D18,
 662 respectively), (D) GPVI-Fc N72Q (monomeric, measured molecular weight is at 98040-
 663 98530 Da due to heterogeneous glycosylation in Fc, similar to that based on the sequence
 664 of the monomeric protein, at 95154 Da.) and (E) GPVI-Fc N72Q with D18 (1:1 complex
 665 formation detected, measured molecular weight is at 120641 Da, similar to that based on the
 666 protein sequence of the 1:1 complex, at 117181 Da). Orange diamonds and blue spheres
 667 represent GPVI and Affimer D18, respectively. (F) Molecular model of D18 dimer generated
 668 using AlphaFold. Molecular docking model of (G) M17, (H) D22 and (I) D18 interacting with
 669 GPVI generated using HADDOCK. GPVI is coloured in orange and brown. The regions
 670 interacting with Affimers predicted by HDX-MS are coloured in cyan. Affimers are coloured in
 671 magenta and green. The variable loops that are crucial for interacting with GPVI are
 672 coloured in red.

Figure 1

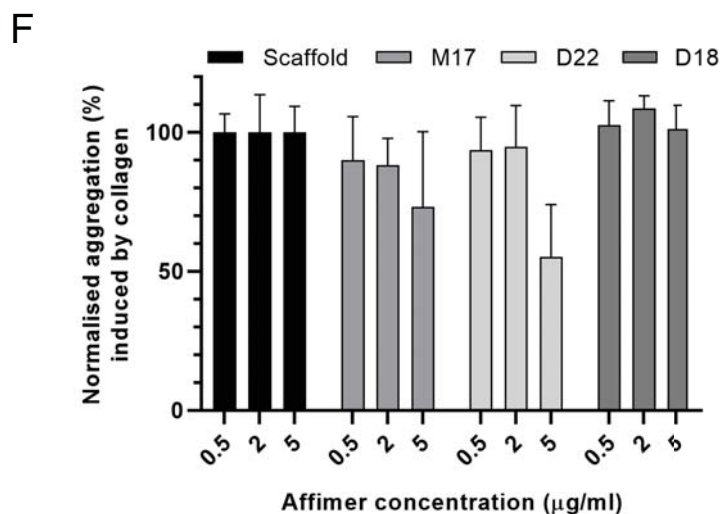
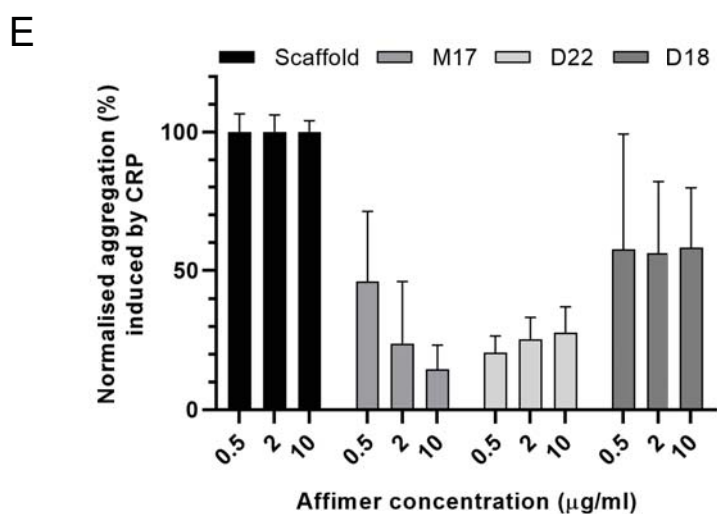
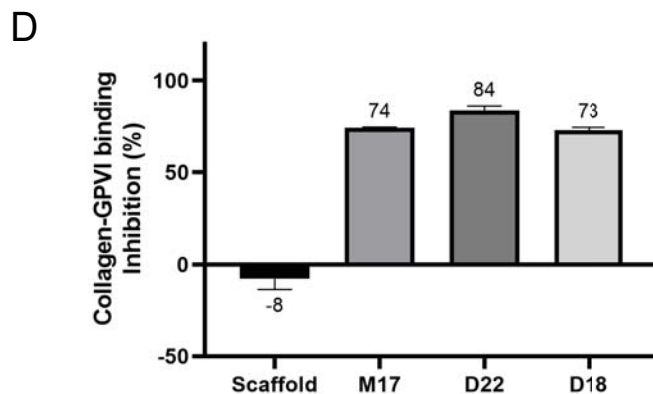
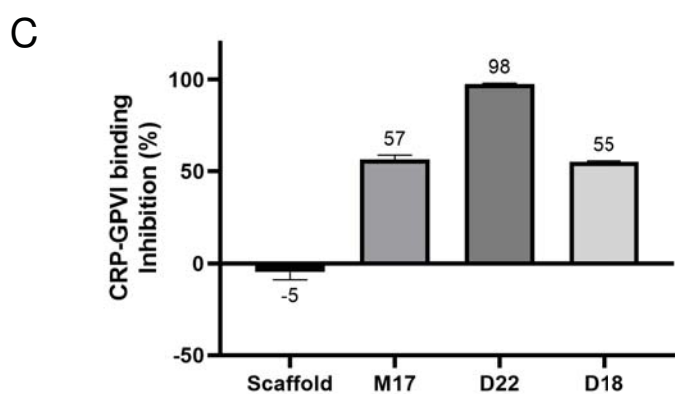
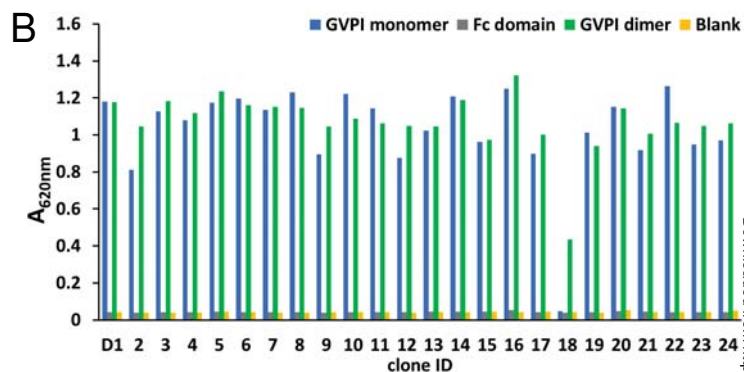
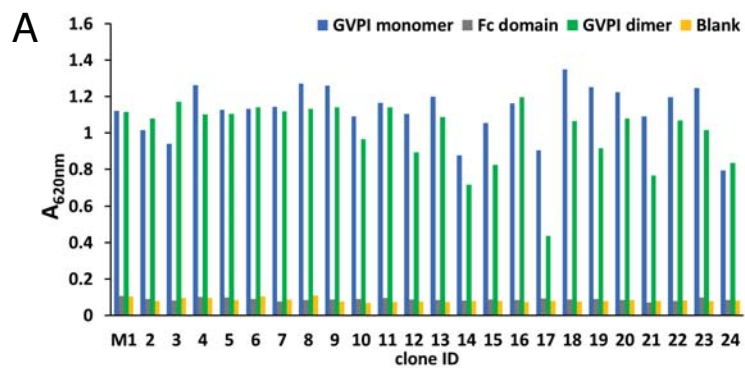


Figure 2

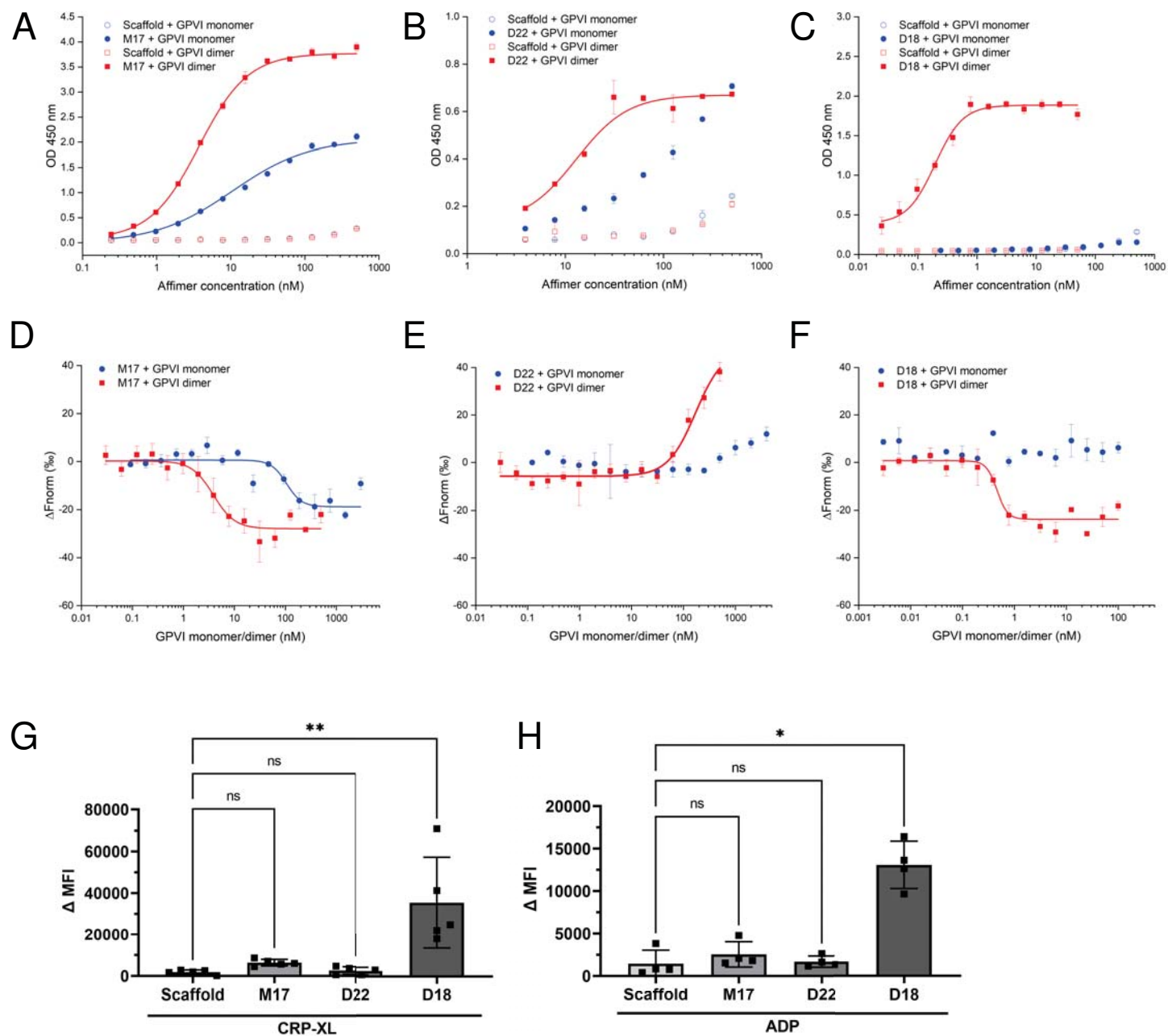
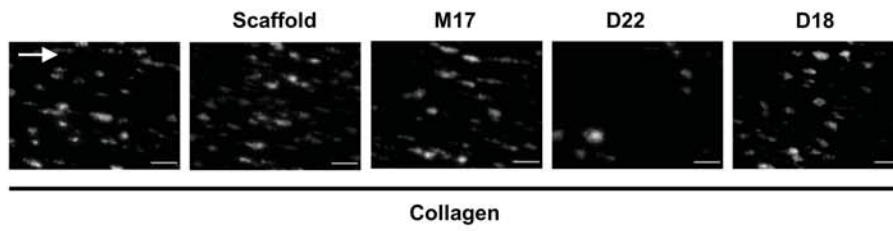
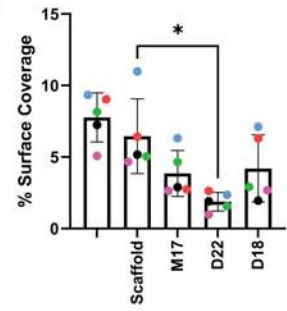


Figure 3

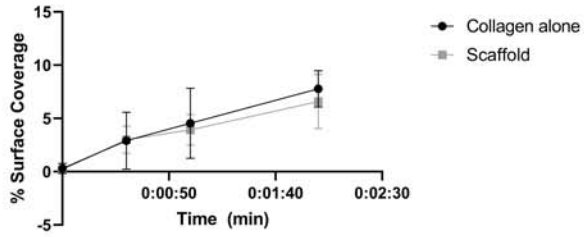
A



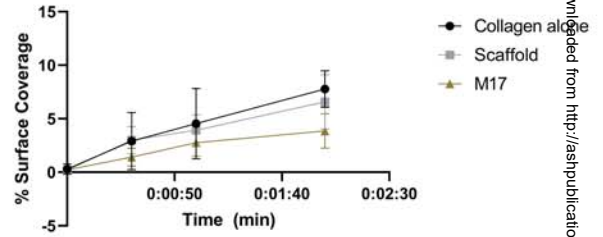
B



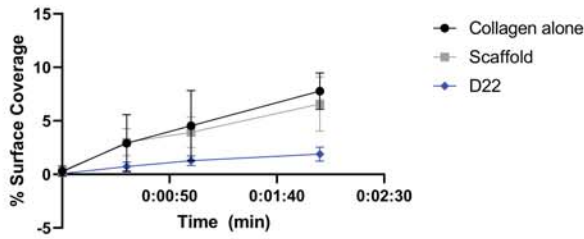
C



D



E



F

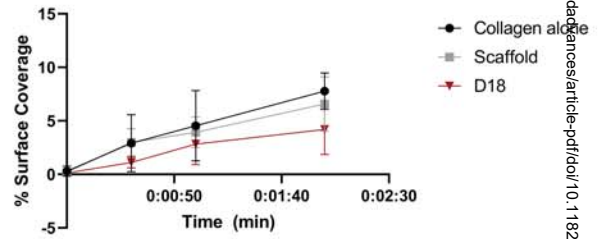


Figure 4

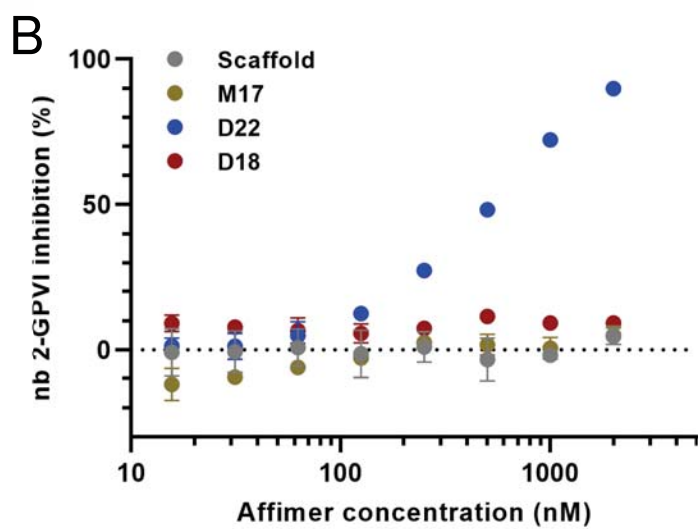
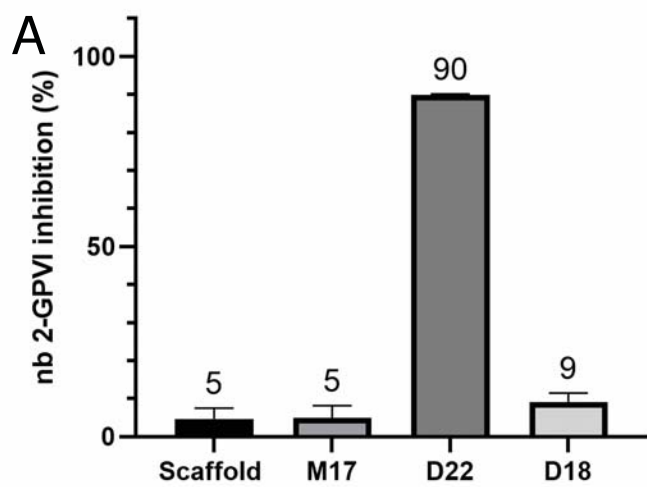


Figure 5

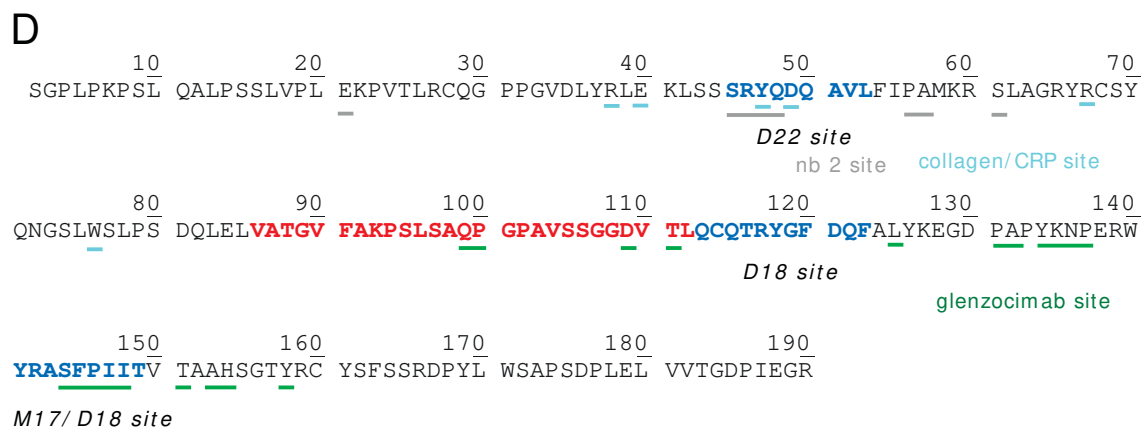
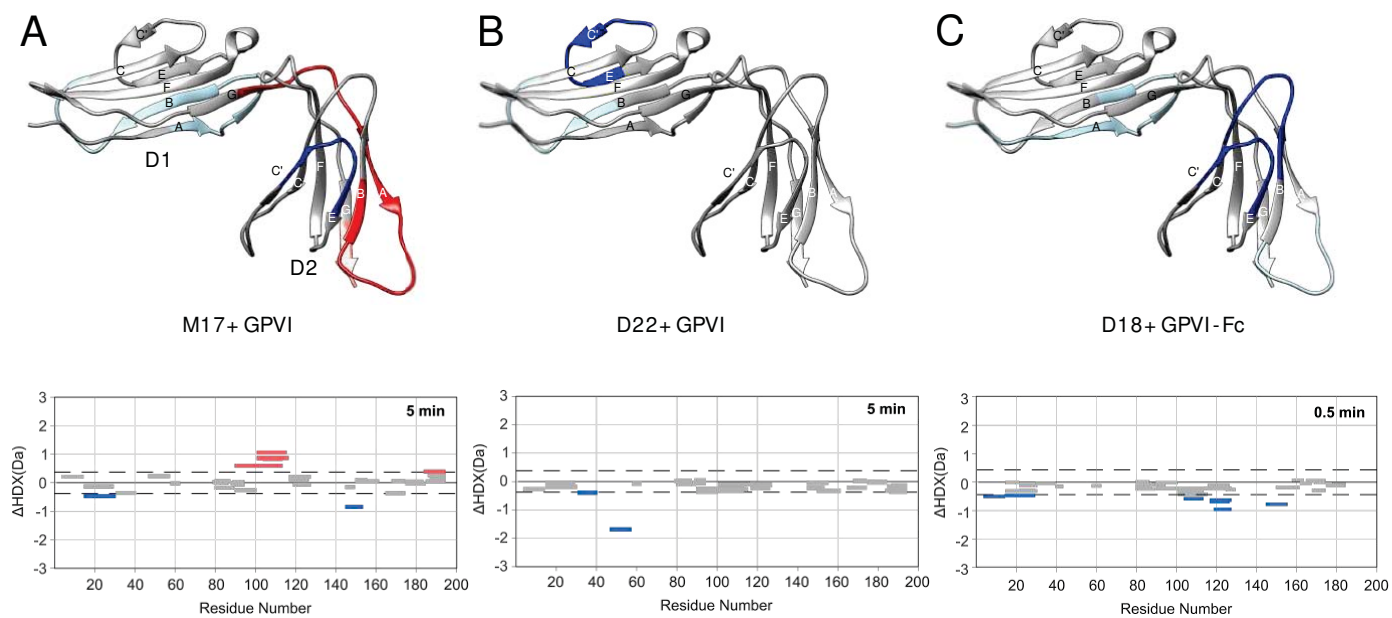


Figure 6

