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Modifying Binding Affinity of Nanobody Caplacizumab to treat Acquired Thrombotic Thrombocytopenic Purpura

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ABSTRACT

Acquired thrombotic thrombocytopenic purpura(aTTP) is a disorder caused by a lack of the ADAMTS13 enzyme, which acts as a blood-clot regulator. Caplacizumab is a nanobody treatment for this disease and interacts with the A1 domain of von Willebrand factor (vWF) to prevent pathological clot formations.

We utilized computational biology tools to predict how mutations will affect the binding affinity and stability of Caplacizumab for interaction with vWF. With these tools, mutations with positive ΔΔG values indicated beneficial outcomes. Certain mutations only had a positive effect on affinity or stability. Single-mutation affinity predictions identified that residues G67, S64, E66, etc, had a positive effect on affinity, highlighting it as a mutational hotspot. However, D63K, D63R, D63I, and E66M had the most stable and positive effects on the nanobody. Residues with a positive effect are located in complementarity-determining region 2 (CDR2), making it a key engineering hotspot. Beneficial mutations were then modeled using Alpha Fold. Overall, results showed a clear trade-off between binding affinity and stability, with few residues maintaining both values with positive ΔΔG.

INTRODUCTION

ATTP is a life-threatening autoimmune disorder in which unusual blood clotting in small vessels causes dangerously low platelet counts and can lead to organ failure. VWF is a multimeric protein responsible for clot formation. Typically, the enzyme ADAMTS13 breaks down vWF but in aTTP, anti-ADAMTS13 antibodies attack the enzyme. Caplacizumab is a nanobody that binds to the A1 domain of vWF is a new treatment for aTTP that reduces clot formation and shortens the time to platelet count recovery. Despite its clinical benefits, Caplacizumab can cause bleeding-related side effects.

The purpose of this project is to try to improve the binding affinity and stability between the Caplacizumab nanobody and the vWF protein to enhance its effectiveness in the treatment of aTTP. Computational tools can predict and simulate how mutations affect protein interaction without wet-lab

screening. For this project, a tiered computational method was utilized, including sequence based prediction tools and structural modeling to identify candidate mutations.

LITERATURE REVIEW

Mechanism of aTTP disease:

The domain architecture of vWF is D1-D2-D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK. (Lenting, 2015) The A1 domain of vWF mediates the interaction with the platelet glycoprotein Ib-IX-V receptor during clot formation (Scully, 2019). The A1 domain will be concealed in the vWF folds while circulating in the plasma through the human body. When the vWF is released, it will assemble into the ultra-large (UL) form that is able to interact with the glycoprotein Ib-IX-V receptor. The UL-vWF multimers bind platelets, leading to microthrombus formation in small vessels. (Peyvandi 2010) The ADAMTS13 enzyme is necessary to maintain a balance between hemostasis, which is the function of stopping bleeding, and thrombosis, the function of blood clotting. (Zander 2015) Congenital TTP (cTTP) is a rare inherited disorder where people are born with an abnormally small amount of the ADAMTS13 enzyme.

Treatments:

Current treatments for aTTP include immunosuppressive drugs, plasma exchange (PEX), and Caplacizumab. Steroids and agents such as Rituximab suppress beneficial parts of the immune system when stopping the autoantibody response, leading to high blood pressure & blood sugar as well as increased infection risk. PEX removes the anti-ADAMTS13 antibodies and replaces the harmful plasma, but it does not prevent disease recurrence in all patients. (Watson, 2024). As discussed below, Caplacizumab is a relatively new solution for treating aTTP by directly blocking the vWF-platelet interaction rather than restoring ADAMTS13 activity, although it has been linked with epistaxis and gingival bleeding. (Elverdi, 2019)

Caplacizumab mechanism:

Nanobodies are the smallest antigen-binding fragments found in certain species such as camelids, llamas, and sharks. Caplacizumab is a humanized, bivalently-linked, variable-domain-only nanobody and immunoglobulin fragment that prevents vWF multimers from connecting to platelets. A X-ray crystal structure revealed that caplacizumab interacts with the A1 domain of vWF as an allosteric inhibitor (Lee, 2021). When it binds there, Caplacizumab stabilizes the vWF in an inactive state, preventing platelet adhesion. In the HERCULES trial, 145 patients with aTTP were divided to be given a placebo or Caplacizumab combined with plasma exchange over 30 days. The results showed that patients treated with Caplacizumab had a shorter median time to return to normal platelet count and 67% lower chance of relapsing with TTP than the patients given a placebo. (Scully, 2019) However, people from the caplacizumab group are more likely to experience side effects of bleeding than patients from the placebo group. The Post-HERCULES (NCT02878603) trial is a phase three, ongoing study to test the efficacy and safety of open-label Caplacizumab in patients with aTTP. In this trial, 31 patients experienced an exacerbation(recurrence of aTTP) during the double-blind treatment. 28 of them were on a placebo, while

only three were on Caplacizumab. The median time to platelet count response (≥150 × 109 /L) was 3.49 days. (Knoebl, 2020). Caplacizumab became the standard treatment for aTTP based on the positive results from the HERCULES study and the TITAN study. Despite this success, Caplacizumab's binding interface with vWF has not been fully optimized and works in a different way than scientists expect.

OBJECTIVES AND HYPOTHESIS

The primary objective of this study was to identify amino acid substitutions within the CDRs of Caplacizumab that are predicted to increase its binding affinity and stability. Another objective of this study is to determine whether mutations identified to have a positive effect on Caplacizumab by different tools would support one another's findings. It was hypothesized that mutations to residues at the interface between the two proteins will have a positive effect by improving binding affinity, thereby destabilizing the A1 domain of vWF to better prevent pathological clot formation in aTTP patients. This hypothesis is addressed and our scientific process is described below, starting with the Methods section which outlines the step-by-step workflow used to identify and validate mutations. Then, the Results section presents our findings at each stage of our experimentations and the trends within the data. The Discussion shows our interpretations of the data and how it applies to the mechanism of the nanobody and the overall bioengineering field. Lastly, the Limitations and Conclusion consist of our reflection upon this experiment.

METHODS

To identify candidate mutations that can improve the binding affinity or stability of Caplacizumab, we used a tiered computational workflow. (Figure 1) We began with the crystal structure of the Caplaciumab-vWF A1 domain complex, and generated all possible substitutions across all three CDRs, which were identified from the reported literature. (Lee 2021) The coordinates for the Caplacizumab bound to the A1 domain of the vWF were downloaded from the protein database under accession number 7eow. Then BioSig tools, including mCSM_PPI, mmCSM_PPI, and mCSM_stability were used to predict the effect of each substitution/mutation on binding affinity and overall protein stability. The raw data inputs were manually examined, and we found the key residues that had a positive effect on affinity or stability, or, in the case of PPI, had a recurrence for the mutation. These residues were manually mutated into the different chain sequences for Chain B, which is Caplacizumab. These chain sequences, along with Chain A, the vWF chain, were inputted into Alphafold, to evaluate whether the mutations were supported by the models of the protein.

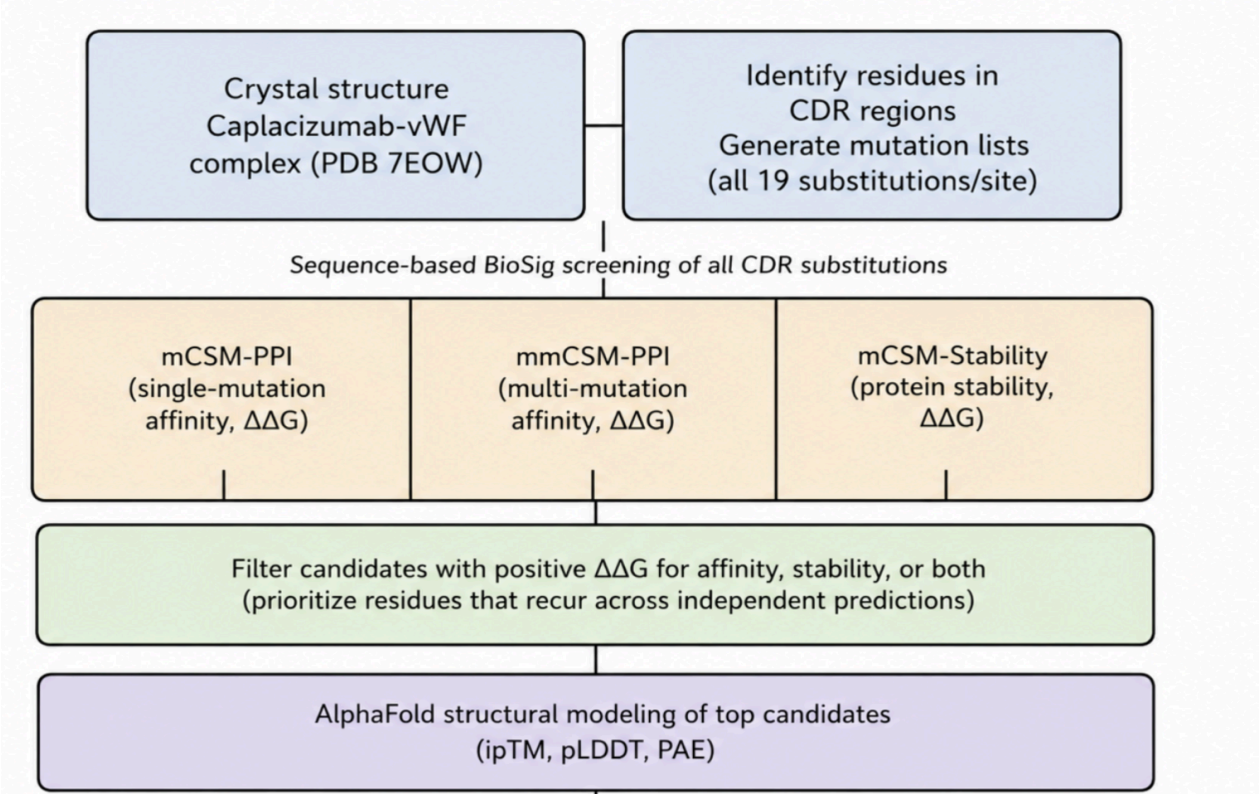

Figure 1. Overview of the tiered computation workflow used to screen and validate candidate Caplacizumab mutations.

BioSig-mCSM_PPI

mCSM_PPI is a tool that predicts the affinity of single-point mutations on the interaction between Caplacizumab and the vwf A1 domain. The tool looks at the atom environment of the mutation site and compares it to a large database of known interactions to guess the energy change. Inputs for this tool include wild-type structure of vWF bound to Caplacizumab (PDB: 7eow) and provided text file mutation lists for each of the three different CDRs, where each residue was mutated to every possible amino acid, in a mutation format (e.g., Chain A, Residue 45, Arginine to Alanine). The output is the Delta Delta G (ΔΔG) value, where positive values indicate the mutation increased the binding affinity between Caplacizumab and the vWF. Negative means the mutation destabilizes the bonding, and positive means the mutation stabilizes the bonding.

BioSig-mmCSM_PPI

mmCSM_PPI is a tool that predicts the effects of systematic multiple mutations on the interactions between Caplacizumab and the A1 domain of vWF. The "mm" stands for Multiple Mutations. It predicts the combined effect of a group of mutations happening at once. This is crucial because sometimes two mutations work together to create a result that neither could do alone. Similar to the single-version, but it also considers the distance and synergy between the multiple mutation sites across the whole interface.

The inputs for this tool are a 3D structure of the 7eow and a list of mutations (e.g., A45G; B12L; C88W). The outputs are ΔΔG values for the entire set of changes and Confidence scores for the prediction.

BioSig-mCSM_Stability

The mCSM_Stability tool analyzes and provides the predictions of protein stability due to mutations. It looks at the internal scaffolding of the protein, which includes hydrogen bonds, salt bridges, etc, and predicts if the mutation will break the internal structures. The inputs include a 3D structure of the single protein and the point mutation. Outputs are ΔΔG values. Negative value is bad/destabilizing, while positive is good/more stable for the drug.

Alphafold:

The Alpafold tool predicts complex formation. Mutations that were positive for affinity, stability, and positive for both were inserted into the protein sequence and supplied in Alphafold predictions for vWF (chain A) and Caplacizumab sequences (chain B). Predictions were assessed by the ipTM, plDDT, and PAE values.

RESULTS

Caplacizumab is a humanized single-domain antibody (VHH or “nanobody”) derived from camelid heavy-chain–only antibodies. Structurally, it consists of a single immunoglobulin variable domain of about 130 amino acids, making it substantially smaller than a conventional IgG antibody. The molecule contains the characteristic VHH β-sandwich fold formed by antiparallel β-strands connected by flexible loops, with antigen recognition mediated primarily through three CDRs (Figure 1). The crystal structure of Caplacizumab bound to the vWF A1 domain shows that this interaction is stabilized by an extensive network of salt bridges, hydrogen bonds, and van der Waals contacts with all three CDRs.

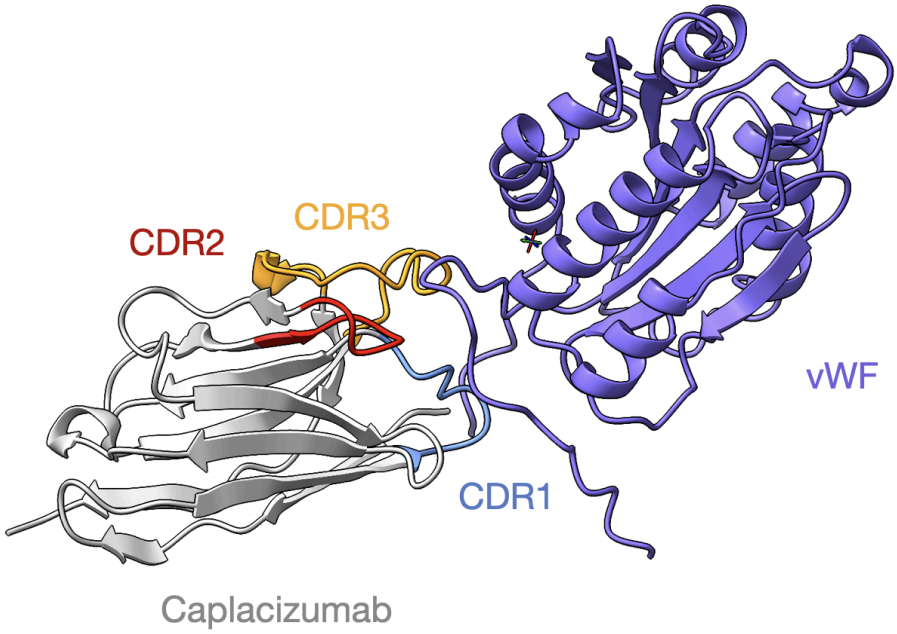

Figure 2. X-ray crystal structure of vWF bound to Caplacizumab (PDBL 7EOW). vWF is colored in purple and caplacizumab is colored in grey. The three CDRs are colored in blue, red, and orange.

The crystal structure of caplacizumab bound to the A1 domain of vWF (PDB ID:7EOW) revealed that the interaction is held together through extensive salt bridges, hydrogen bonds, and vanderwaal contacts involving the three CDRs in the nanobody structure. In order to identify additional changes to the CDRs that enhance the therapeutic potential of caplacizumab, mutations were screened in silico and assessed for changes to its binding affinity to vWF and overall protein stability. The BioSig prediction tools, mCSM-PPI, mmCSM-PPI, and mCSM-stability, were used, starting with the crystal structure of caplacizumab bound to the A1 domain of vWF (PDB ID:7EOW). In silico analysis yielding positive ΔΔG value for either affinity or stability indicated an improvement, whereas negative ΔΔG values indicated poorer affinity or stability.

Single-mutation effects on binding affinity:

The Single-mutation affinity predictions identified that residues G67, G57, D63, G101, S58, S64, E66, T59, and G56 had a positive effect on affinity. All residues were found in CDR 2, except for G101, which was found in CDR 3 (Figure 2).

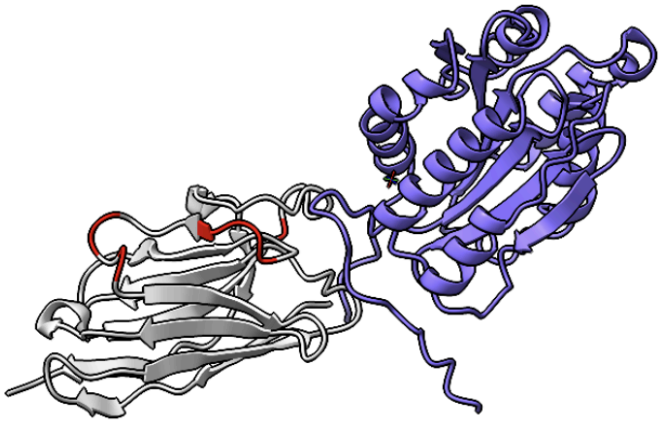

Figure 3. Single mutations mapped onto Caplacizumab with predicted improvements to binding affinity. Caplacizumab is colored in gray and the vWF is colored in purple. The red residues represent the CDR sequences. All labeled residues are in CDR 2 except for G101.

Single-mutation effects on stability:

Even though these residues had a high predicted affinity, many of the designs had mutations that were independently destabilizing in the mCSM-PPI analysis. This could be because the mutations can cause strain, clashes, or folding penalties that are not shown in the affinity-only prediction models.
We utilized the mCSM stability tool to test and predict how different CDR sequences affect the nanobody's stability. S31, T55, S58, T59, D63, S64, E66, V102, R103, E105, D106 were residues that had a positive effect on the stability. The residues listed were identified from all three different CDR files (Figure 3). S31 was found in CDR1, T55, S58, T59, D63, S64, and E66 are found in CDR2, and V102, R103, E105, D106 are all found in CDR 3.

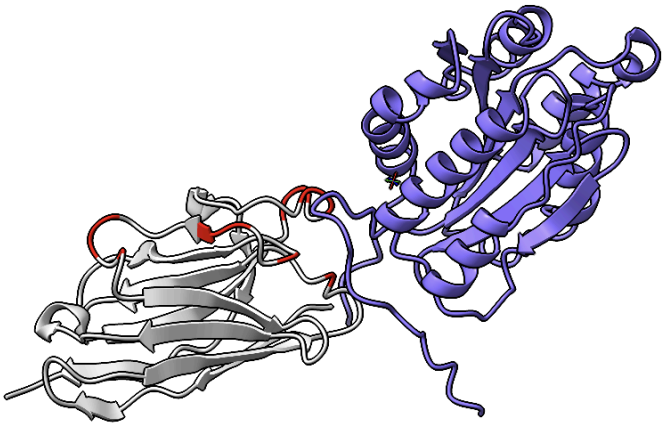

Figure 4. Single mutations mapped onto Caplacizumab with predicted improvements to protein stability. The grey chain is the Caplacizumab chain, interacting with the purple chain which is the vWF chain. Residues labeled in red are predicted to be candidate residues crucial to the bonding of vWF and Caplacizumab.

Single-mutation comparison of effects on binding affinity and stability:

When we compared affinity and stability for the single mutations, we narrowed it down to certain mutations of interest. D63 and E66 are residues that have a positive affinity and positive stability. Residues including D63K, D63R, D63M, D63I, and E66M produced modest but consistent gains in both binding affinity and positive stability ΔΔG values. These residues are partially solvent-exposed and can tolerate charge/hydrophobic substitution without disrupting the stability of the nanobody fold. G67, G57, G101, S58, S64, T59, and G56 had positive affinity but negative results for stability. S31, T55, S58, T59, S64, V102, R102, E104, and D106 were residues that had positive stability but negative results for affinity. Specifically R102A, and E104A are salt bridges at the interface of the nanobody and vWF. By comparing the affinity and stability predictions, the most balanced single-mutation candidates were D63K, D63R, D63M, and E66M. There was a consistent trade off since most residues that had a positive ΔΔG for affinity had a negative ΔΔG for stability besides for D63 and E66.

Residue #	Mutation(s)	ΔΔG affinity	Computational Tool	ΔΔG stability	Computational tool
G67	H W Y F D C E	1.237 1.141 1.139 1.083 0.964 0.938 0.933	mCSM_PPI	-0.622 -1.04 -0.648 -0.803 -0.363 -0.832 -0.475	mCSM_stability
G57	H W Y F D C E	1.011 1.061 1.048 0.904 0.69 0.667 0.666	mCSM_PPI	-0.325 -1.018 -0.697 -0.902 -0.248 -0.894 -0.395	mCSM_stability
D63	H K	0.843 0.598	mCSM_PPI	0.265 0.447	mCSM_stability

	I L R	0.455 0.455 0.379		0.203 0.203 0.635	
G101	H W Y F	0.737 0.488 0.484 0.419	mCSM_PPI	-1.354 -1.497 -1.093 -1.158	mCSM_stability
S58	H	0.598	mCSM_PPI	-1.356	mCSM_stability
S64	H M	0.576 0.455	mCSM_PPI	-0.984 -0.005	mCSM_stability
E66	M	0.549	mCSM_PPI	0.316	mCSM_stability
T59	H	0.545	mCSM_PPI	-1.175	mCSM_stability
G56	H	0.502	mCSM_PPI	-0.669	mCSM_stability

Table 1. Single point mutations with positive effects on affinity, using mCSM_PPI and their corresponding predicted effect on the stability, using mCSM_stability.

Residue #	Mutations	ΔΔG stability	Computational tool	ΔΔG affinity	Computational tool
S31	D E	0.425 0.4	mCSM_stability	-1.543 -1.553	mCSM_PPI
T55	D E	0.422 0.336	mCSM_stability	-2.084 -2.074	mCSM_PPI
S58	D E	0.177 0.08	mCSM_stability	-0.324 -0.411	mCSM_PPI
T59	D E	0.336 0.395	mCSM_stability	-0.358 -0.433	mCSM_PPI
D63	C H I K L	0.004 0.265 0.203 0.447 0.203	mCSM_stability	0.061 0.843 0.455 0.598 0.455	mCSM_PPI

	M N P Q R S T V Y	0.156 0.209 0.124 0.212 0.635 0.106 0.224 0.124 0.063		0.873 -1.007 0.282 -0.406 0.379 -0.734 -0.521 0.282 0.183	
S64	D E	0.143 0.081	mCSM_stability	-0.208 -0.251	mCSM_PPI
E66	M	0.316	mCSM_stability	0.549	mCSM_PPI
V102	R	0.184	mCSM_stability	-0.605	mCSM_PPI
R103	I L M N P V Y	0.368 0.368 0.045 0.122 0.231 0.231 0.028	mCSM_stability	-1.084 -1.084 -0.439 -0.838 -1.015 -1.015 -0.635	mCSM_PPI
E105	H I K L M N P Q R S T V	0 0.095 0.545 0.095 0.192 0.342 0.015 0.351 0.616 0.206 0.319 0.015	mCSM_stability	-0.423 -0.729 -1.401 -0.729 -0.047 -2.228 -0.923 -1.797 -1.402 -2.372 -2.189 -0.923	mCSM_PPI
D106	I	0.112	mCSM_stabilit	-1.03	mCSM_PPI

	L M	0.112 0.483	y	-1.03 -0.362	

Table 2. Single point mutations with positive effects on stability, using mCSM_stability and their corresponding predicted effect on the affinity, using mCSM_PPI.

Multi-mutation effects on binding affinity:

The predictions for multiple mutations were measured using predictions from the mmCSM_PPI tool. Based on that, we discovered that certain combinations of residues were more common. E105Y; D106W, E105Y; D106Y, A104W; R28F, E105Y; A104W, E105Y; A104Y were the recurring residue combinations from most repeated to least repeated, respectively (Figure 4). However, when these were tested as single mutations, they ended up having a negative ΔΔG value for either stability or affinity. For example, E105 and D106 had a negative effect on stability, while A104 and R28 had a negative effect on both, but seemed to be a combination mutation with E105 and D106. E105, D106, and A104 are all located in CDR 3 and R28 is not a part of the three CDRs.

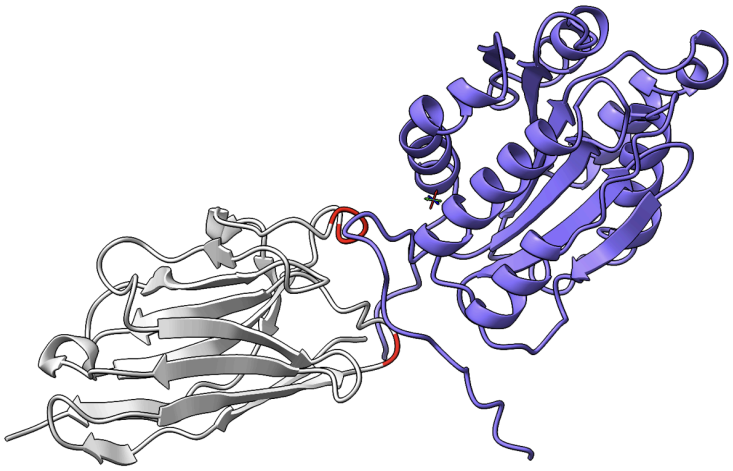

Figure 5. Residues involved in the most frequently recurring multi-mutation combinations, mapped onto the Caplacizumab structure. All residues shown are located in CDR3, except R28.

Pair	Frequency
B E105Y;B D106W	7X
B E105Y;B D106Y	4X

B A104W;B R28F	3X
B E105Y;B A104W	4X
B E105Y;B A104Y	5X

Table 3. Multiple Mutation Frequency Table

Predicting structural changes in vWF-caplacizumab interaction by AlphaFold:
The AlphaFold server tool was used to model and simulate the interaction between the vWF and the modified Caplacizumab nanobody. Residues D63K, D63I, E66M, G101H, G101Y, G101F, G101W, T55D, R102E, R102A, E104R, E104A were independently inserted into Chain B, which is the wild-type Caplacizumab. Chain A, which is the vWF chain, and the mutated Chain Bs were inputs for Alpha Fold. Figures 6-15 show the Alphafold models for all residues inputted into the server.

Outputs were evaluated based on ipTM, plDDT confidence level, or predicted positional error. ipTM stands for the interface predicted template modelling score, which shows the accuracy of the interaction between different chains in the alphaFold prediction. Values under 0.6 suggest there is not a confident interaction predicted, while values over 0.8 indicate a confident predicted interaction. The pIDDT score provides a measure for overall structure prediction of sequence elements within the overall structure and colored according to higher scores. Lastly, the predicted position error matrix is a metric of how confident each residue is predicted with respect to the other residues included in the prediction. Low predicted error for residues located at the interface suggest confidence in the predicted protein-protein interaction. Our positive control was the original wild type caplacizumab chain, which had an ipTM score of 0.85 consistent with an experimentally validated interaction and structure determined by X-ray crystallography (Figure 6).

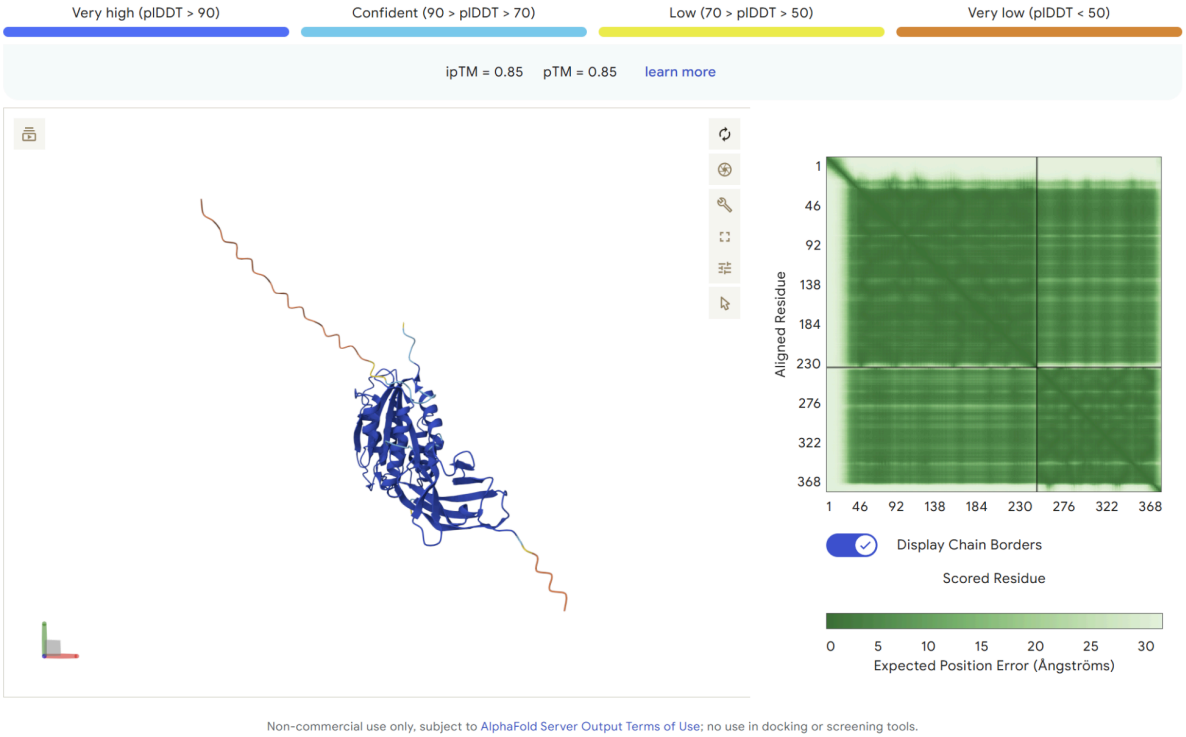

Figure 6. AlphaFold prediction of wildtype Caplacizumab bound to vWF.(positive control) ipTM = 0.85, indicating high confidence in the predicted interaction; pLDDT scores exceed 90 across the folded A1 domain and nanobody domain, and predicted aligned error is low.

Further, we used a mutation of R102A,E104A which corresponds to two residues that participate in salt bridges at the interface as a negative control with corresponding iPTM score of 0.13 consistent with a perturbed interaction (Figure 6).

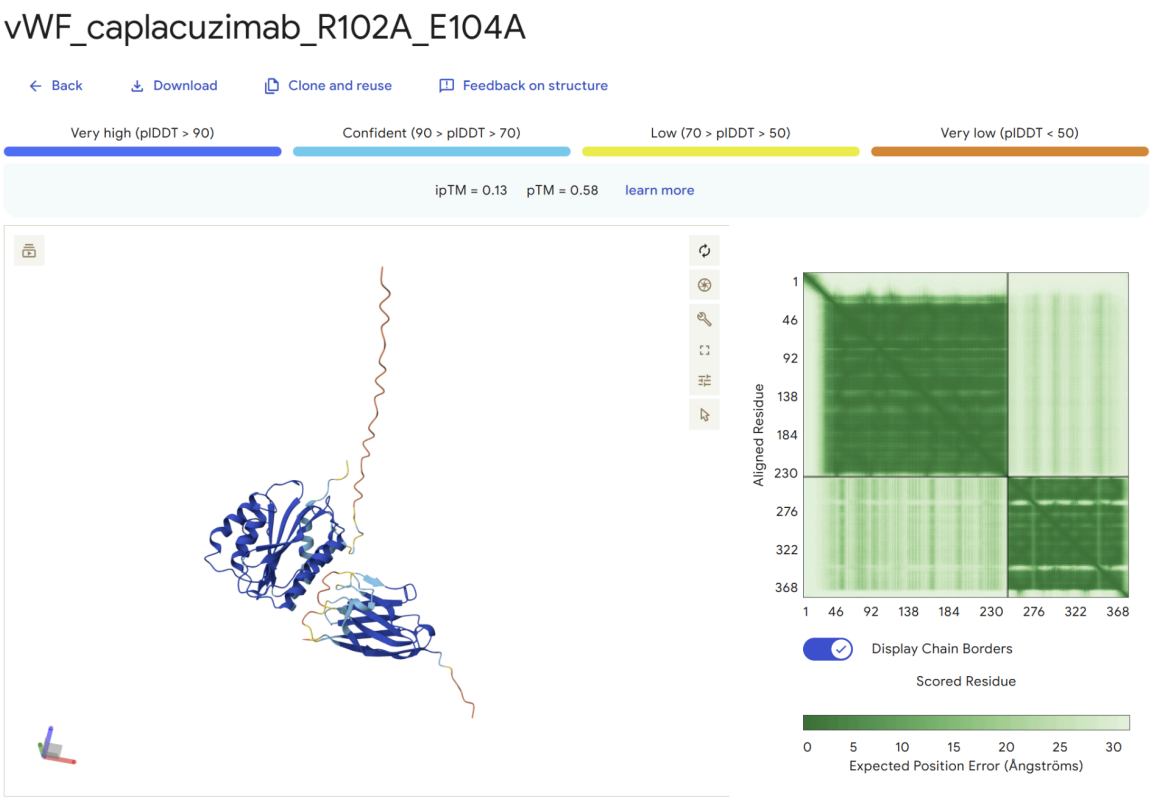

Figure 7. AlphaFold prediction of Caplacizumab R102A, E104A bound to vWF. iPTM score of 0.13 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

We then compared the Alphafold predictions for Caplacizumab variants including the residue changes identified through our computational screen. Despite showing positive changes in either binding affinity or stability, T55D (Figure 7), D63K (Figure 8), G101H (Figure 9), G101Y (Figure 10), G101W (Figure 11), and G101F (Figure 12) failed to predict a confident interaction between Caplacizumab and vWF as indicated by low confidence in the variable regions of the nanobody and low iPTM scores.

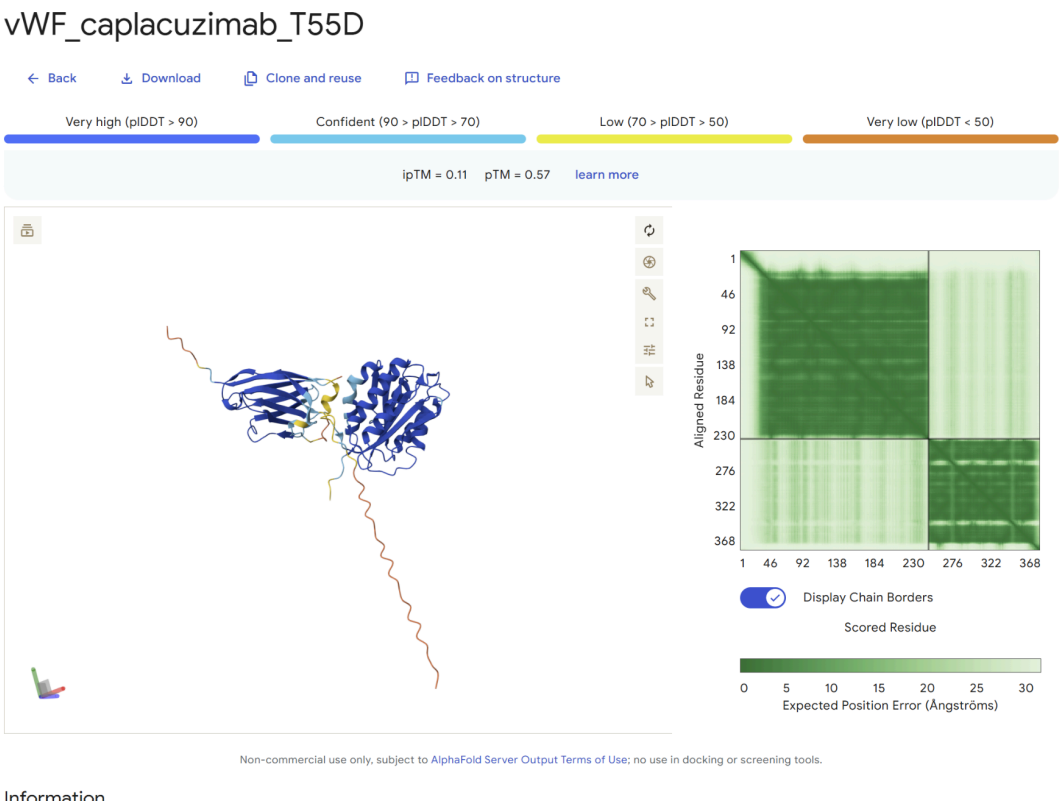

Figure 8. AlphaFold prediction of Caplacizumab, T55D bound to vWF. iPTM score of 0.11 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

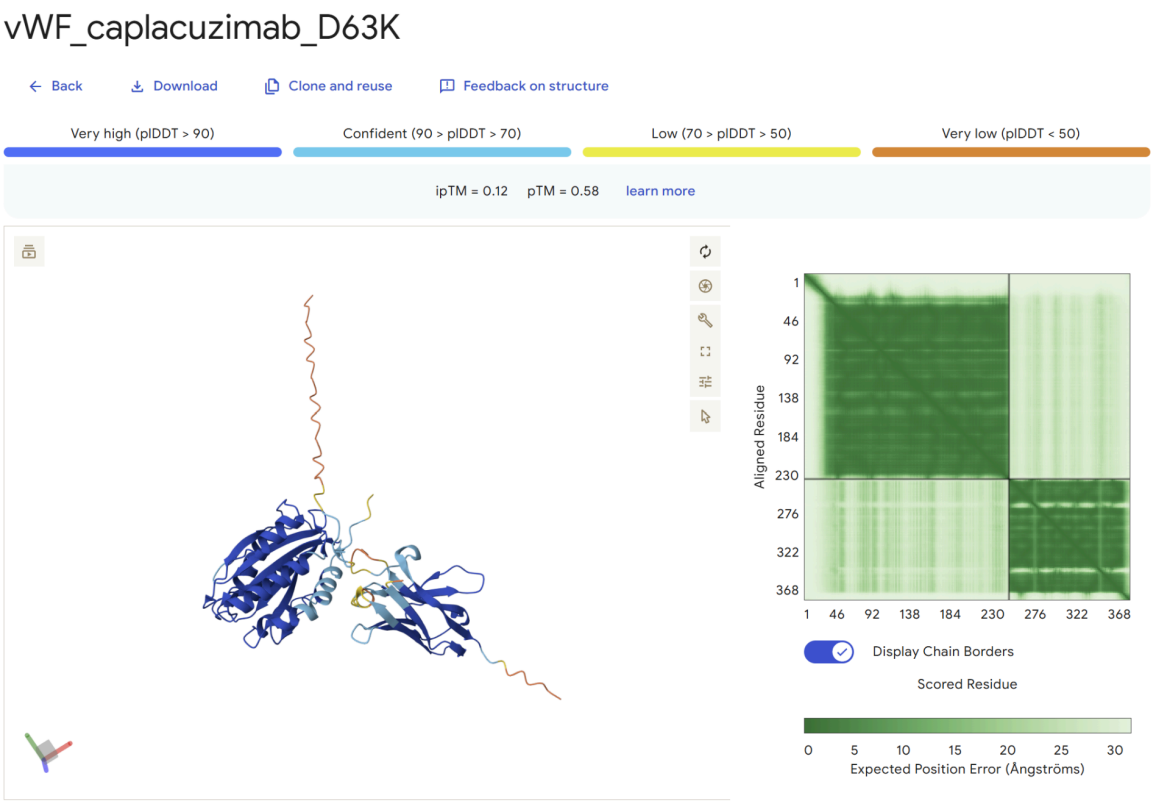

Figure 9. AlphaFold prediction of Caplacizumab, D63K bound to vWF. iPTM score of 0.12 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

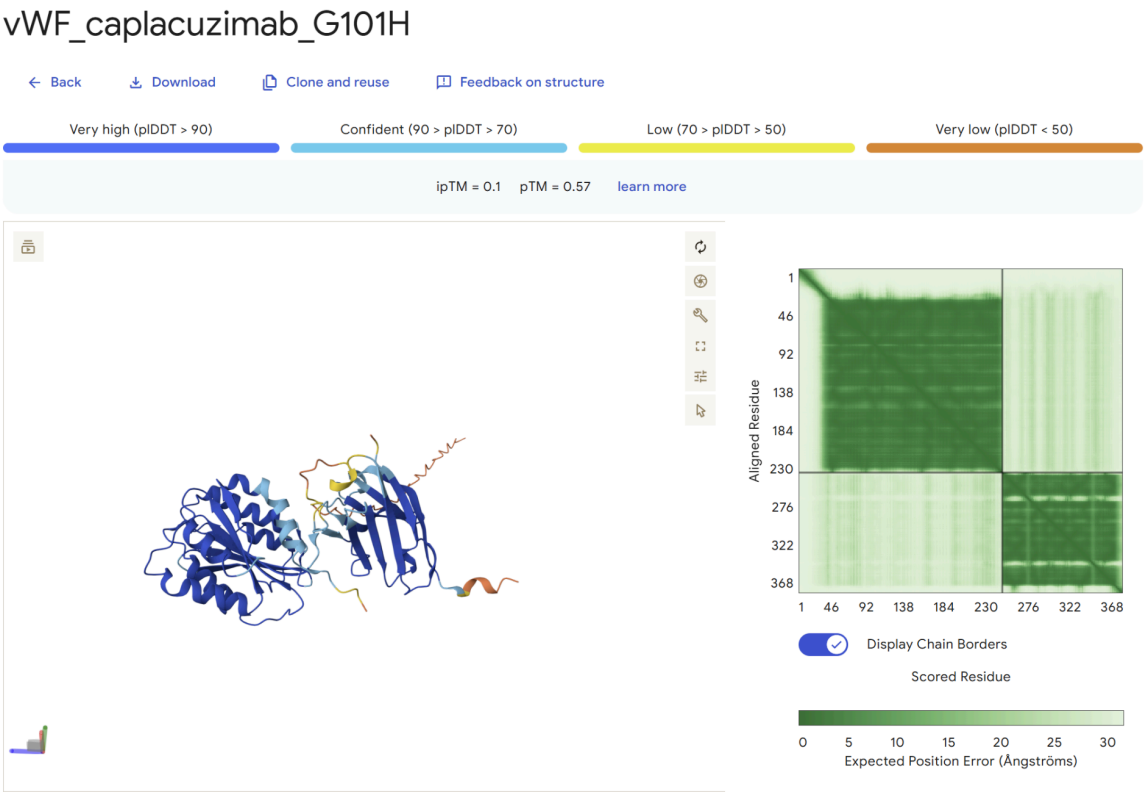

Figure 10. AlphaFold prediction of Caplacizumab, G101H bound to vWF. iPTM score of 0.1 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

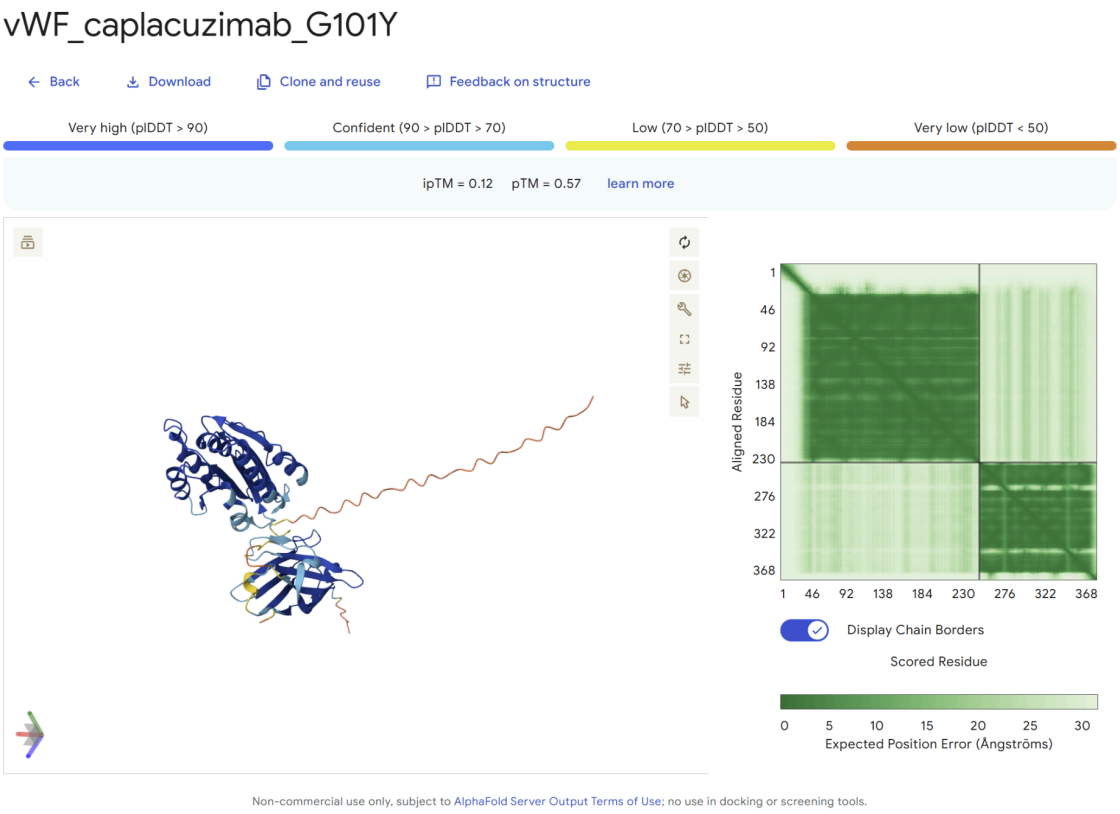

Figure 11. AlphaFold prediction of Caplacizumab, G101Y bound to vWF. iPTM score of 0.12 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

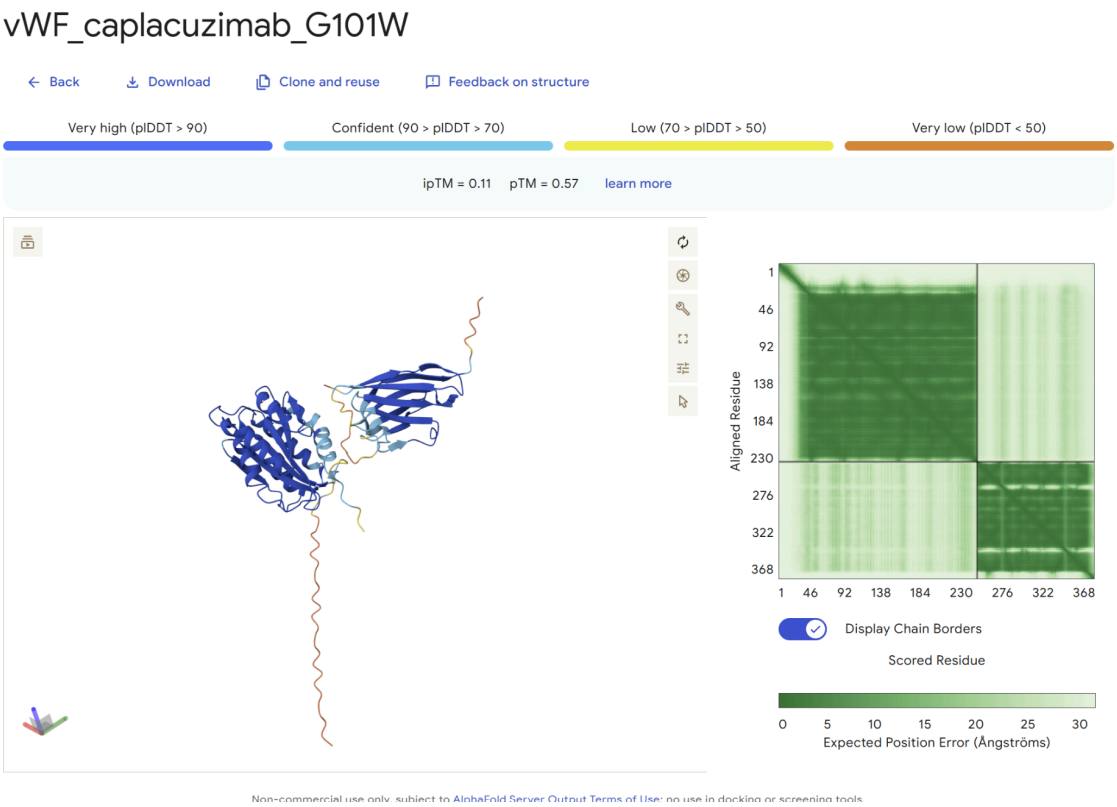

Figure 12. AlphaFold prediction of Caplacizumab, G101W bound to vWF. iPTM score of 0.11 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

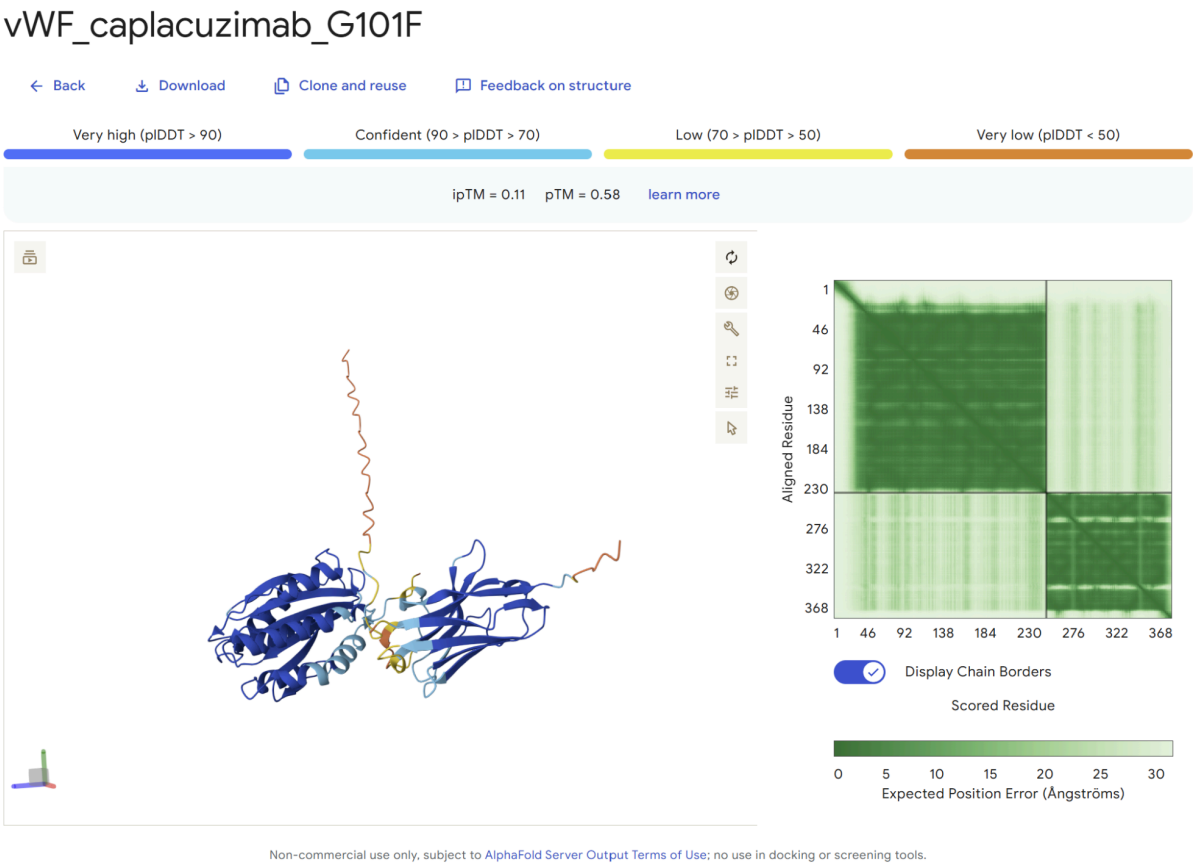

Figure 13. AlphaFold prediction of Caplacizumab, D63K bound to vWF. iPTM score of 0.11 indicates low confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and <70 for the CDRs of Caplacizumab. Expected position error is high for Caplacizumab relative to vWF.

However, mutation of D63I in Caplacizumab yielded an iPTM score of 0.79 indicating a predicted interaction (Figure 13). Further, the predicted position error for residues for both chains was low and the majority of the structure had plDDT scores greater than 90 (Figure 13).

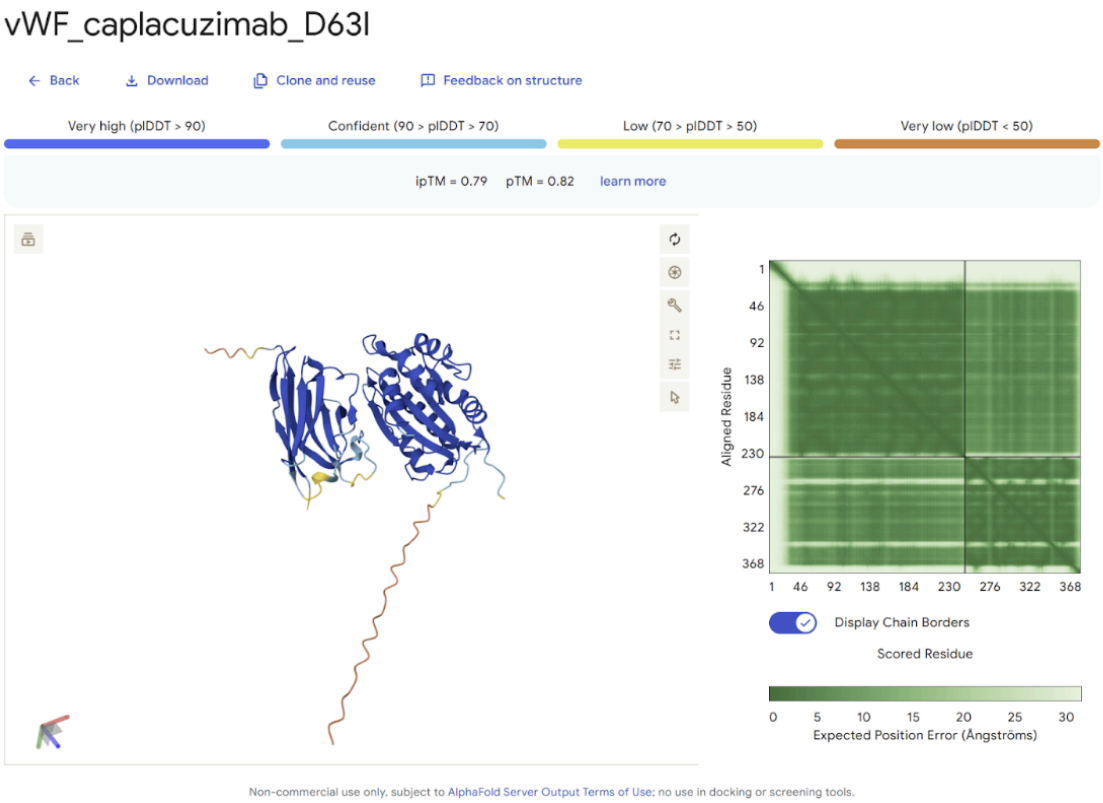

Figure 14. AlphaFold prediction of Caplacizumab, D63I bound to vWF. iPTM score of 0.79 indicates high confidence in vWF-Caplacizuman interaction. plDDT scores are greater than 90 in the folded A1 domain of vWF and >70 for the CDRs of Caplacizumab. Expected position error is low for Caplacizumab relative to vWF.

Interestingly, the D63I variant predicts an alternative interaction surface to the previously determined crystal structure (Figure 14). Normally, D63 is located distal to the protein-protein interaction surface with Caplacizumab. The D63I alphafold prediction now shows vWF and Caplacizumab interacting at an interface including D63I, suggesting that this mutation may affect the overall complex formation and mechanism of action of Caplacizumab.

Variant	ipTM	Interpretation
Wild-type Caplacizumab	0.85	High confidence (positive control)
R102A-E104A	0.13	Low confidence (negative control)
T55D	0.11	Low confidence
D63K	0.12	Low confidence

G101H	0.10	Low confidence
G101Y	0.12	Low confidence
G101W	0.11	Low confidence
G101F	0.11	Low confidence
D63I	0.79	High confidence

Table 4. Alpha-fold interface confidence (ipTM) scores for the wild-type complex, controls, and candidate Caplacizumab substitutions.

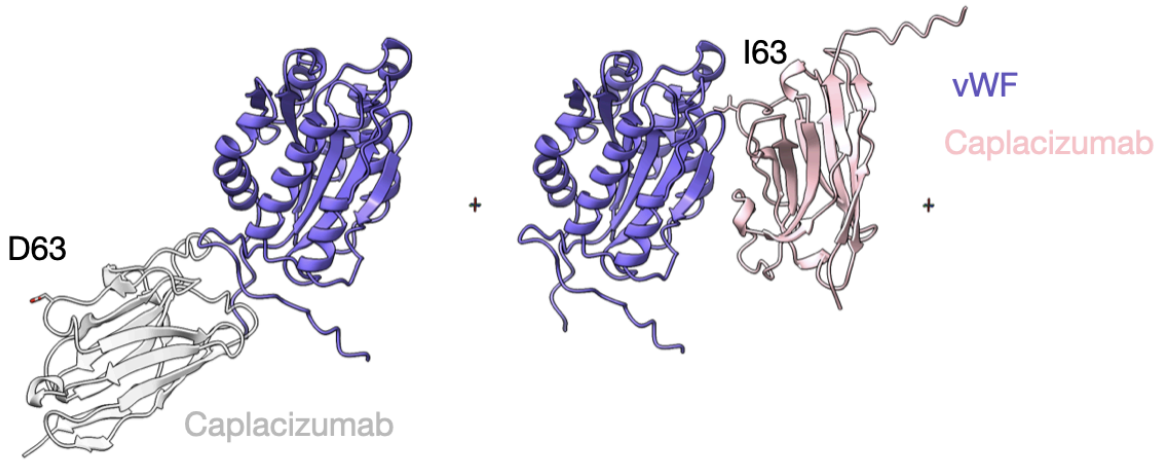

Figure 15. Comparison of Caplacizumab-vWF interactions for wildtype and D63I variants. The crystal structure (PDB:7EOW) shows D63 located away from the vWF-Caplacizumab interface (left). The Alphafold prediction of I63 Caplacizumab shows vWF interacting close to the mutation (right).

DISCUSSION

Throughout this process, the results show that modifying Caplacizumab involves a trade off between its binding affinity with vWF and its structural stability. It also shows that CDR2, specifically residue D63, is an engineering hotspot within the nanobody. Even though aromatic substitutions at the binding interface enhanced the predicted affinity, it negatively affected the stability of the nanobody as a whole.

This study has shown that the mCSM tools, which are sequence based ΔΔG predictions, did not match with the structure-based Alphafold modeling. Five of the six BioSig prioritized candidates such as T55, D63 and G101 were put into Alphafold. However the results contradicted between the tools. This is likely because each method measures different units. Tools from BioSig measure energetic effects using the surrounding atomic environment while AlphaFold evaluates the entire geometry of the protein. In future experiments, a similar tiered process would be used but tools that measure similar units would be used to avoid differing results. The sequence based screening and structural confirmation is similar to strategies used in most nanobody experiments to narrow down candidate mutations before performing wet-lab experiments.

These mutations are all found in CDR2, which was identified as a mutational hotspot, considering other mutations with positive affinity/stability values were also located there. D63 is a mutation that tolerates various substitutions. This could be because positively charged side chains, such as K and R, that may form new electrostatic interactions to reduce intramolecular repulsion within the nanobody. Furthermore, hydrophobic substitutions such as M and I may enhance local packing and reduce solvent exposure without clashes in the stability/folds. These residues preserve the flexibility of the side chains and do not increase the volume of the residues. D63 also returned an Alphafold confidence score that was similar to the one of the Wild-type protein. Furthermore, the predicted structure also suggested that an alternative Caplacizumab-vWF interface was not present in the crystal structure, in which the mutated CDR2 loop makes direct contact with vWF. Since D63 is normally positioned away from the interface, this creates the possibility that D63I does not simply reinforce the existing wild-type binding mode, but instead introduces a new contact surface that changes how Caplacizumab engages vWF.

Based on all of this, we can confirm that D63I is the best candidate in improving Caplacizumab's affinity for vWF while preserving nanobody stability, and better demonstrates the value of combining sequence-based and structure-based computational tools to prioritize mutations for antibody and nanobody engineering.

LIMITATIONS

The study was limited by a lack of access to wet lab tools and relied heavily on predictions made by computational tools. In order to validate the findings from this study, recombinant proteins could be purified with the identified mutations and assessed for changes to affinity with vWF by biophysical methods such as isothermal calorimetry or surface plasmon resonance. Further, a cellular assay could be developed using a disease model cell line to compare the efficacy of wild-type Caplacizumab for treatment of aTTP versus Caplacizumab harboring the mutations identified here. Caplacizumab would be tested to see if it can bind to the A1 domain of vWF using assays. The results of this test would be compared with the computational predictions involving binding affinity to find out how accurately the in silico model is predicting the effects of caplacizumab and its mutations. If the introduced mutations

function similarly or better than Caplacizumab, they could further be assessed for improvements in pharmacokinetic properties that may perform better in patients.

CONCLUSION

An in silico mutational analysis was performed to engineer and modify the caplacizumab nanobody to have improved binding to vWF while maintaining stability. The BioSig prediction tools, mCSM-PPI, mmCSM-PPI, and mCSM-stability were used, starting with the crystal structure PDB ID:7EOW. The single-point mutations were evaluated along with modifications to the CDRs at the interface. Data from affinity and stability prediction tools showed that caplacizumab can be engineered to have higher affinity for vWF. Even though some substitutions at the binding interface enhanced the predicted affinity, it negatively affected the stability of the nanobody as a whole. This shows a clear trade-off in affinity and stability during the engineering of the caplacizumab nanobody. Positive ΔΔG affinity predictions indicated better binding, while negative values indicated reduced affinity. This was the same for stability predictions. The residue D63I affects the protein-protein interaction surface on caplacizumab and may change its mechanism of action with VWF. This residue in CDR2 was a key engineering hotspot since multiple substitutions improved both affinity and stability. The most balanced single mutation residue candidates, when run through Alpha Fold, did not yield ipTM, plDDT, and PAE scores that indicated better performance than caplacizumab while multi-mutations on residues like R102 and E104(that play an important role in surface interaction) showed a negative effect on interaction in alphaFold. If we were to continue this experiment in the future, we would test mutations on interaction through biophysical methods, test mutations on treatment of aTTP to conclude if they work through the same mechanism as Caplacizumab, and test if the mutations perform better than Caplacizumab.

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