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Article

Novel aptamer to von Willebrand factor A1 domain (TAGX-0004) shows total inhibition of thrombus formation superior to ARC1779 and comparable to caplacizumab

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Running title: Novel DNA aptamer targeting VWF A1 domain

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ABSTRACT

von Willebrand factor (VWF) is a blood glycoprotein that plays an important role in platelet thrombus formation through interaction between its A1 domain and platelet glycoprotein Ib. ARC1779, an aptamer to the VWF A1 domain, was evaluated in a clinical trial for acquired thrombotic thrombocytopenic purpura (aTTP). Subsequently, caplacizumab, an anti-VWF A1 domain nanobody, was approved for aTTP in Europe and the United States. We recently developed a novel DNA aptamer, TAGX-0004, to the VWF A1 domain; it contains an artificial base and demonstrates high affinity for VWF. To compare the effects of these three agents on VWF A1, their ability to inhibit ristocetin- or botrocetin-induced platelet aggregation under static conditions was analyzed, and the inhibition of thrombus formation under high shear stress was investigated in a microchip flow chamber system. In both assays, TAGX-0004 showed stronger inhibition than ARC1779, and had comparable inhibitory effects to caplacizumab. The binding sites of TAGX-0004 and ARC1779 were analyzed with surface plasmon resonance performed using alanine scanning mutagenesis of the VWF A1 domain. An electrophoretic mobility shift assay showed that R1395 and R1399 in the A1 domain bound to both aptamers. R1287, K1362, and R1392 contributed to ARC1779 binding, and F1366 was essential for TAGX-0004 binding. Surface plasmon resonance analysis of the binding sites of caplacizumab identified five amino acids in the VWF A1 domain (K1362, R1392, R1395, R1399, and K1406). These results suggested that TAGX-0004 possessed better pharmacological properties than caplacizumab *in vitro* and might be similarly promising for aTTP treatment.

Introduction

von Willebrand factor (VWF) is a large multimeric plasma glycoprotein that plays an essential role in tethering circulating platelets to damaged endothelial cells.[1] Interaction between the VWF A1 domain and the platelet glycoprotein (GP) Ib receptor leads to rapid development of VWF-rich platelet thrombi, thus preventing further bleeding.[2] Although normal amounts of VWF are usually helpful in hemostasis reactions, several VWF-mediated diseases, such as acute coronary syndrome,[3, 4] cerebral infarction,[5] and thrombotic thrombocytopenic purpura (TTP),[6] demonstrate remarkably elevated plasma levels of VWF. Inhibiting VWF activity by blocking its A1 domain has recently been demonstrated to be an attractive therapeutic target in these arterial thromboses.[7-10]

Caplacizumab (Ablynx), an anti-VWF A1 humanized nanobody,[11, 12] was approved by the European Medicines Agency in 2018 and by United States Food and Drug Administration in 2019 for treatment of acquired TTP (aTTP). The anti-VWF A1 aptamer ARC1779 (Archemix) was developed as an antithrombotic agent for use in patients with aTTP, and it showed sufficient inhibitory effects on VWF activity without any severe bleeding events in a randomized control trial.[13] However, ARC1779 has not yet been approved for the treatment of VWF-mediated thrombosis. We recently generated a novel DNA aptamer, TAGX-0004, that contains the artificial hydrophobic base 7-(2-thienyl)imidazo[4,5-b]pyridine (Ds); this aptamer targets human VWF A1 with very high affinity (K_D = 61.3 pM) and specificity.[14]

In this study, we compared TAGX-0004, ARC1779, and caplacizumab in terms of their inhibitory effects on VWF activity, by performing conventional platelet aggregation assays, ristocetin- and botrocetin-induced platelet aggregation assays (RIPA and BIPA, respectively), and shear-stress–induced aggregation assays using the

microchip flow chamber system (T-TAS®).[15] To assess the affinity and binding sites of the two aptamers, we performed biophysical interaction analysis and alanine scanning mutagenesis of the VWF A1 domain. In addition, we analyzed the binding sites of caplacizumab using surface plasmon resonance (SPR).

Methods

Detailed of materials and methods are presented in the Supplemental information.

Sources of nucleoside, oligonucleotides, nanobody, and protein

The artificial nucleoside triphosphate (dDsTP) was synthesized as described previously.[16, 17] The oligonucleotides TAGX-0004 and ARC1779 (without polyethylene glycol [PEG]) were synthesized by and purchased from GeneDesign, Inc. (Osaka, Japan). The oligonucleotide sequence of TAGX-0004 (as Rn-DsDs-51mh2)[14] and that of ARC1779[18] were reported previously. The anti-human VWF nanobody caplacizumab (as TAB-234) was purchased from Creative-Biolabs (NY, USA). The recombinant human VWF A1 domain protein was purchased from U-Protein Express BV (Utrecht, Netherlands), and used for the measurement of binding affinity to anti-human VWF A1 agents using SPR.

Alanine-scanning mutagenesis

Based on previous reports that described the interaction between VWF A1 and GPIb,[19] botrocetin,[20] or ARC1172,[21] we designed 16 alanine-substituted mutants of the human VWF A1 domain (R1287, K1312, R1334, R1336, K1348, K1362, F1366, K1371,

E1376, R1392, R1395, R1399, K1406, K1423, R1426, and K1430). These mutant proteins were generated using a cell-free expression system (Taiyo Nippon Sanso Corporation, Tokyo, Japan).

Platelet Aggregation Test (PAT)

PAT was performed with PRP313M aggregometer (TAIYO Instruments INC, Osaka, Japan). Aggregation inducing substances and their final concentration were as follows; ristocetin (1.5 mg/mL), botrocetin (1.0 μg/mL), collagen (4 μg/mL), epinephrine (1.0×10^{-4} M) and adenosine diphosphate (ADP) (1.0×10^{-5} M).

Total thrombus formation analysis system (T-TAS)

The T-TAS® (Zacros, Fujimori Kogyo Co. Ltd., Tokyo, Japan) is a micro-chip flow-chamber device used to visually and quantitatively analyze thrombus formation in whole blood samples under various blood flow conditions.[15] Whole blood samples with each anti-VWF A1 agent were applied into collagen I coated micro-chip (PL chip). Subsequently, the thrombus formation in the capillaries and an increase in flow pressure were observed.

Electrophoresis mobility shift assay (EMSA)

The binding abilities of the two aptamers to the human VWF A1 domain and its alanine-substituted mutants were analyzed by EMSA. Each aptamer (final concentration of 100 nM) was mixed with VWF A1 (final concentration of 0 to 800 nM) in binding buffer and incubated at 37 °C for 30 min, then subjected to 8% native PAGE in 0.5× TBE buffer for 50 min at room temperature (200 V/cm). The aptamer-VWF A1

complexes were detected as a shift band, and the band patterns were detected by SYBR Gold. The dissociation rate (K_D) was calculated by Scatchard plot analysis.

Surface plasmon resonance

Competition assays with the two aptamers were performed by SPR analysis using Biacore T200 (GE Healthcare UK Ltd., Little Chalfont, UK), as described previously.[14] We also performed SPR analysis to investigate the binding site of the VWF A1 domain to caplacizumab.

Structure models of the VWF A1 domain

Three-dimensional (3D) structure models of the VWF A1 domain were visualized with the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, USA).

Ethical statement

This study was approved by the ethics committee of Nara Medical University. Written informed consent was obtained from the individual who donated plasma for use in this study.

Results

Inhibitory effects on platelet aggregation

The inhibitory effects of TAGX-0004, ARC1779, and caplacizumab under static conditions were analyzed by PAT. Figure 1 shows representative results of the analyses of the three types of plasma. All three agents showed inhibition activities in both RIPA

and BIPA. However, the inhibitory effects on platelet aggregation differed between the three agents. In RIPA, the 80% maximal inhibitory concentration (IC_{80}) of TAGX-0004 was 50 nM, whereas that of ARC1779 was 500 nM and that of caplacizumab was 50 nM; thus TAGX-0004 blocked VWF function via the A1 domain approximately 10 times more potently than ARC1779, and exhibited a similar potency as caplacizumab. In BIPA, the IC_{80} of TAGX-0004 was 50 nM, whereas that of ARC1779 was 500 nM and that of caplacizumab was 50 nM, respectively; thus TAGX-0004 blocked VWF function approximately 10 times more potently than ARC1779 showing a similar potency as caplacizumab. None of the three agents inhibited platelet aggregation induced by collagen, epinephrine, or ADP (data not shown).

Inhibitory effects on thrombus formation

To assess the inhibitory effect of the three agents on platelet thrombus formation, we performed T-TAS. In this study, complete inhibition was defined as an increase in flow pressure from baseline of no more than 10 kPa. TAGX-0004, ARC1779, and caplacizumab were analyzed three times each, and Figure 2 shows representative flow pressure curves. Both TAGX-0004 and caplacizumab prevented thrombus occlusion under the flow condition at 50 nM. In contrast, ARC1779 did not demonstrate complete inhibition, even at a concentration of 1000 nM. These results indicated that TAGX-0004 inhibited platelet thrombus formation at least 20 times more potently than ARC1779 under high shear stress, and with a similar potency as caplacizumab.

Biophysical interaction analysis with EMSA

Biophysical interaction analysis with EMSA was performed four times for each

aptamer. Figure 3 shows that the affinity of TAGX-0004 to VWF A1 domain (K_D = 2.2 ± 0.9 nM [n = 4]) was approximately 16-fold higher than that of ARC1779 (K_D = 35.5 ± 1.5 nM [n = 4]).

Alanine scanning mutagenesis with EMSA

Figure 4 shows the results of EMSA using 16 alanine-mutated VWF A1 with TAGX-0004 (A) or ARC1779 (B). First, we analyzed nine mutants as shown in the left panels. Based on the results of this initial analysis, we then analyzed additional seven mutants as shown in the right panels. Of these, 10 VWF A1 mutants (K1312A, R1334A, R1336A, K1348A, K1371A, E1376A, K1406A, K1423A, R1426A, and K1430A) formed shifted bands representing complexes between an aptamer and VWF A1, as well as between wild-type (WT) VWF A1 and each aptamer. In the remaining six mutants, the intensity of the complexed bands was decreased, and the intensity of the unbound aptamer bands was increased. Of these mutants, R1395A and R1399A showed decreased intensity in both TAGX-0004 and ARC1779 band complexes.

While the binding affinity to ARC1779 was significantly decreased in R1287A, K1362A, and R1392A, that to TAGX-0004 was decreased in F1366A. These findings suggested that R1395 and R1399 were essential residues for binding to both aptamers, and that R1287, K1362, and R1392 probably contributed to binding to only ARC1779, while F1366 was required for binding to TAGX-0004. Our data indicated that both aptamers bound to the VWF A1 domain, even though the residues necessary for this binding were partially different, and these differences might affect their VWF inhibitory activity.

Competition assay with Biacore

To compare the binding sites of the aptamers to the VWF A1 domain, a competition assay was performed by SPR using Biacore. Biotinylated TAGX-0004 was immobilized on the sensor chip SA. Then, VWF A1 and a competitor aptamer (TAGX-0004 or ARC1779) as an analyte was injected into the chip. The results of a self-competition assay using TAGX-0004 showed that the amount of VWF A1 binding to the chip decreased depending on the concentration of competitor TAGX-0004 (Supplementary Figure 1). ARC1779 also dose-dependently decreased the response unit value. These results indicated that TAGX-0004 and ARC1779 bound to the overlapped region on the VWF A1 domain. Moreover, the competitive ability of ARC1779 against immobilized TAGX-0004 on the chip surface was lower than that of TAGX-0004 against TAGX-0004. These results were consistent with those of EMSA and indicated that the binding affinity of TAGX-0004 for VWF A1 was higher than that of ARC1779.

VWF A1 domain binding site of caplacizumab

We performed SPR analysis to investigate the VWF A1 domain binding site of caplacizumab. After immobilizing caplacizumab on the sensor chip CM5, 16 alanine-substituted mutants of VWF A1 were analyzed (Figure 5). The relative binding amount of WT was defined as 100%. The binding amount of each mutant was expressed as the relative ratio compared to that of WT. When a relative ratio of 80% was set as the cut-off, five mutants were judged as positive. These results indicated that these five residues (K1362, R1392, R1395, R1399, and K1406) were likely to be the binding sites of caplacizumab.

Comparison of VWF A1 domain binding sites of TAGX-0004, ARC1779, and caplacizumab

Figure 6 shows 3D structure models of the VWF A1 domain. The amino acid residues shown in red indicate the essential residues for binding to each aptamer or caplacizumab. The residues shown in yellow were not involved in binding. Of the 16 alanine-substituted VWF A1 mutants, EMSA predicted that three amino acids (F1366, R1395, and R1399) were binding sites for TAGX-0004, as shown in red in Figure 6 left. The remaining 13 amino acid residues are shown in yellow. As for ARC1779, five amino acid residues (R1287, K1362, R1392, R1395, and R1399) played an important role in binding to VWF A1, as shown in red in Figure 6 middle. Of these, R1395 and R1399 were amino acids shared by both aptamers. Further, using Biacore we identified five amino acid residues in the VWF A1 domain (K1362, R1392, R1395, R1399, and K1406) that were binding sites to caplacizumab, as shown in red in Figure 6 right. Of these, K1406 was unique to caplacizumab. The differences in VWF A1 domain binding sites suggested that these aptamers or nanobody might have unique effects on platelet aggregation and thrombosis formation.

Discussion

VWF plays a pivotal role in the initial phase of platelet thrombus formation under high shear stress through the interaction between its A1 domain and platelet GPIb.[1, 2] The binding site of the VWF A1 domain is usually cryptic and prevents spontaneous binding to platelets. High shear stress of blood flow induces conformational changes in

VWF and exposes the VWF A1 domain. Therefore, the association between VWF A1 and platelet GPIb usually occurs under high shear stress.[2]

Inhibition of VWF A1 domain binding to platelet GPIb could prevent the development of platelet thrombus formation that causes cardiac infarction, cerebral infarction, and TTP. TTP results from the formation of platelet thrombi in the microvasculature due to a deficiency of ADAMTS13, a VWF-cleaving protease.[22, 23] Therefore, preventing VWF A1 binding to platelet GPIb could be a promising therapeutic target for TTP. For this purpose, caplacizumab was evaluated for its anti-thrombotic effects in a phase 2 clinical trial (the TITAN study)[11] and a phase 3 clinical trial (the HERCULES study).[12] These studies reported a therapeutic effect of caplacizumab on aTTP, including faster recovery of platelet counts, fewer plasma exchange sessions, and shorter hospital stays. Bleeding event was reported as a common adverse event in patients treated with caplacizumab compared with patients without it.[12] In the TITAN and HERCULES study, 36 and 72 patients of aTTP were treated with caplacizumab and 19 (54%) and 46 (65%) patients had bleeding events, respectively. The most common adverse events were epistaxis and gingival bleeding, neither of which generally required treatment.

Nucleic acid aptamers are single-strand DNA or RNA molecules that can form 3D structures capable of specifically binding to proteins or other cellular targets. They are superior to existing antibody products in terms of their specificity, manufacturing cost, relatively small size, and non-immunogenicity. Pegaptanib (Macugen®, Pfizer) is the only aptamer approved by FDA in 2004 for the treatment of wet age-related macular degeneration blocking vascular endothelial growth factor.[24] As of June 2019, there are no aptamer for the patients with coagulation or thrombotic disorder is approved.

However, some aptamers targeting coagulation factors, ARC 1779 against VWF A1, NU172 (ARCA Biopharma) against factor IIa, and REG1/REG2 (Regado Biosciences) against factor IXa, stepped into clinical trials.[13, 25-27] ARC1779 was developed to target the VWF A1 domain and was evaluated in a phase 2 clinical trial in patients with aTTP.[13] Unfortunately, the recruitment of patients in this trial was terminated without completing the study due to sponsor-related financial issues, but seven patients with aTTP received combined therapy with intravenous ARC1779 injections and plasma exchange.[13] The trial showed no severe adverse events such as bleeding, even in patients with aTTP who had severe thrombocytopenia.[13] A study in healthy volunteers confirmed that this aptamer had an antithrombotic effect and did not cause severe bleeding events.[28]

TAGX-0004 is a novel DNA aptamer that targets the VWF A1 domain and contains Ds, an artificial nucleic acid. TAGX-0004 was obtained using modified SELEX (systemic evolution of ligands by exponential enrichment) methods incorporating the Ds base.[14] Since the Ds base has no complementary base in nature, Ds-containing DNA aptamers can have unique 3D structures. The high hydrophobicity of the Ds base may contribute to significantly high binding affinity to target proteins. In fact, the results of EMSA showed that the TAGX-0004 binding affinity for VWF (K_D = 2.2 ± 0.9 nM) was 16-fold higher than that of ARC1779 (K_D = 35.5 ± 1.5 nM) (Figure 3). However, the K_D values in this study might be underestimated in comparison with previous reports; the K_D value of TAGX-0004 was shown to be 61.3 pM by a SPR assay,[14] and the K_D value of ARC1779 was 2 nM in a RI-labeled assay.[18] Previous results also indicated that the binding affinity of TAGX-0004 was much higher than that of ARC1779.

The binding affinity of aptamers to VWF A1 domain might affect the interaction

between VWF and platelets. Although both TAGX-0004 and ARC1779 inhibited the platelet aggregations which were induced by ristocetin and botrocetin, the minimum concentration of TAGX-0004 necessary for inhibition was significantly lower than that of ARC1779 in both RIPA and BIPA (Figure 1). PAT analysis revealed that TAGX-0004 could block VWF function via the A1 domain at least 10 times more strongly than ARC1779. T-TAS also revealed that TAGX-0004 was superior to ARC1779 at inhibiting VWF function (Figure 2). In addition, compared with caplacizumab, TAGX-0004 showed equally effective inhibition against thrombosis formation under various blood flow conditions. Moreover, TAGX-0004 has a unique mini-hairpin DNA structure that offers benefits in pharmaceutical applications,[14] specifically by conferring resistance to degradation by nucleases. This structure should extend the half-life of this molecule *in vivo*.

As described above regarding the adverse effects of caplacizumab treatment, there are still concerns regarding bleeding caused by anti-VWF antagonists. Although anti-VWF agents have demonstrated superior safety profiles in this regard compared with anti-platelet agents, we should prepare measures to quickly treat bleeding that occurs during aptamer treatment. We speculate that if a neutralizing agent comprising a DNA sequence that is partially complementary to that of a specific aptamer is prepared, it may serve as an effective antidote as introduced in the literature.[29] In the case of TAGX-0004, such partial DNA sequence may contain pyrrole-2-carbaldehyde (Pa), the complementary base pair to Ds.[16] In the past development of antithrombotic aptamer therapeutics, similar approaches of antidote were taken to neutralize the unwanted effect of aptamer, though such aptamers are yet to be not approved.[26] The specific antidote could contribute to control severe bleeding compared with caplacizumab.

Alanine mutagenesis analysis of the VWF A1 domain was performed in this study to determine the binding sites of TAGX-0004, ARC1779, and caplacizumab. The binding sites of ARC1779, but not TAGX-0004 or caplacizumab, were reported previously.[21] Our results showed that the binding sites of these three agents only partially corresponded each other (Figure 6). Huang et al[21] reported that 18 amino acid residues in the VWF A1 domain were binding sites for ARC1172, which has the same fundamental structure as ARC1779. That study did not identify R1399 as a binding site, even though in the present study it was a common binding site for all three agents. Chen et al identified R1399 as one of the key amino acids for the effect of VWF to hemostasis and thrombosis.[30] Interestingly, a hydrophobic amino acid residue (F1366) is required to bind to TAGX-0004, which has two hydrophobic Ds bases. Matsunaga et al previously reported that the number of Ds base (0-2) strongly correlated with binding affinity to VWF A1[14] and we confirmed that Ds-free TAGX-0004 could not inhibit thrombus formation on T-TAS (data not shown). These results indicated that the Ds base interacts directly to the VWF A1 domain and therefore F1366 appears to be an essential and unique binding site of TAGX-0004 to the VWF-A1 domain with hydrophobic interaction (e.g. pi-stacking). Of note, alanine scanning of caplacizumab showed that like ARC1779 and TAGX-0004, both R1395 and R1399 were necessary to bind the VWF A1 domain (Figure 6).

Due to the method limitations, the affinity between the VWF A1 domain and TAGX-0004 or ARC1779 was not analyzed by the SPR method. Since both oligonucleotides and the sensor chip are negatively charged, the K_D value was underestimated when we immobilized the VWF A1 protein on the sensor chip and applied TAGX-0004 or ARC1779 as an analyte. Therefore, we performed alanine

scanning by EMSA for DNA aptamers. In addition, the binding site of caplacizumab to VWF A1 domain was not analyzed with EMSA, which was used to assess TAGX-0004 and ARC1779, since this technique is not suitable for the analysis of proteins such as nanobodies and antibodies. Secondly, ARC1779 was originally PEGylated. However, we used ARC1779 without PEG in this study because we found no obvious differences between ARC1779 with and without PEG using RIPA, BIPA, T-TAS, and EMSA (data not shown).

In addition, caplacizumab was used in clinical study and approved as bivalent nanobody which has two binding sites to the target. On the other hand, two aptamers used in this study were formed as monovalent. It is known that direct comparison of an affinity of monovalent entity with an avidity of bivalent entity is not straightforward. Nevertheless, it is of intriguing that TAGX-0004 has shown comparable inhibitory effect against caplacizumab in the studies we performed, and how bivalent form of TAGX-0004, which is not available though, behaves in the same experiments.

Treatment with caplacizumab has demonstrated a rapid decrease of VWF ristocetin cofactor assay in patients with aTTP and the levels of VWF antigen and factor VIII were also transiently reduced with caplacizumab treatment compared with placebo due to an increased clearance of the caplacizumab-VWF complex.[11] Whether or not TAGX-0004 should demonstrates equivalent effect is yet to study using *in vivo* model. Nevertheless, judging from the binding ability of TAGX-0004 to plasma-derived human VWF confirmed with EMSA (data not shown), it is likely that the aptamer shows similar effect as caplacizumab in aTTP patient.

In conclusion, we confirmed the potency of TAGX-0004 for preventing platelet thrombus formation *in vitro*. Epitope mapping of the binding sites of TAGX-0004

compared with ARC1779 and caplacizumab provided us with information on each molecule's efficacy and characteristics. Caplacizumab is now coming into use as a first-line therapy for aTTP. However, there are still challenges to be overcome with this agent, such as bleeding adverse events and high cost.[31] TAGX-0004 has the potential to overcome these problems and could be developed as a promising drug not only for aTTP, but also for various VWF-mediated thrombotic disorders such as acute coronary syndrome and cerebral infarction.

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Figure legends

Figure 1 The inhibitory effects of TAGX-0004, ARC1779, and caplacizumab on platelet aggregation under static conditions

As shown in the upper left panel, TAGX-0004 completely inhibited ristocetin-induced platelet aggregation (RIPA) at a concentration of 50 nM. In contrast, the concentration required for ARC1779 was 500 nM, and that for caplacizumb was 50 nM. As shown in lower left panel, TAGX-0004 inhibited botrocetin-induced platelet aggregation (BIPA) at a concentration of 50 nM. The concentration required for ARC1779 was 500 nM, and that for caplacizumab was 50 nM.

Figure 2 The inhibitory effects of TAGX-0004, ARC1779, and caplacizumab on platelet thrombus formation under high shear stress

Pressure curves at various concentrations of each agent are shown; curves end at the point of complete microcapillary occlusion. As shown in the upper panel, TAGX-0004 inhibited thrombus formation under high shear conditions at a final concentration of 50 nM. The middle panel shows that ARC1779 did not achieve complete inhibition even at a concentration of 1000 nM. The lower panel shows that caplacizumab completely inhibited thrombus formation at a concentration of 50 nM.

Figure 3 Biophysical interaction analysis of TAGX-0004 and ARC1779 with electrophoresis mobility shift assay (EMSA)

The white arrowhead indicates the complex of wild-type recombinant VWF A1 with an aptamer (TAGX-0004 or ARC1779). The black arrowhead indicates unbound aptamer. Increasing the concentration of wild-type VWF A1 increased the density of complex bands and decreased that of unbound aptamer bands. These experiments were performed four times for each aptamer. The dissociation rates (K_D) of TAGX-0004 and ARC1779 were 2.2 ± 0.9 nM and 35.5 ± 1.5 nM, respectively.

Figure 4 Electrophoresis mobility shift assay (EMSA) using alanine-scanning mutants for analyzing VWF A1 binding to TAGX-0004 and ARC1779

The white arrowhead indicates the complex of VWF A1 with an aptamer (TAGX-0004 or ARC1779). The black arrowhead indicates unbound aptamer. The leftmost lane in each gel (-) represents aptamer only, without a VWF A1 mutant. Using both aptamers, 10 VWF A1 mutants (K1312A, R1334A, R1336A, K1348A, K1371A, E1376A, K1406A, K1423A, R1426, and K1430A) formed complexes with aptamers, as did wild-type (WT) VWF A1. With TAGX-0004, as shown in the top panels, three mutants (F1366A, R1395A, and R1399A, indicated in red letters) demonstrated decreased densities of complex bands and increased densities of unbound bands, indicating that these amino acids were important for the binding of VWF A1 to TAGX-0004. With ARC1779, as shown in the lower panels, five mutants (R1287A, K1362A, R1392A,

R1395A, and R1399A) demonstrated decreased densities of complex bands and increased densities of unbound bands.

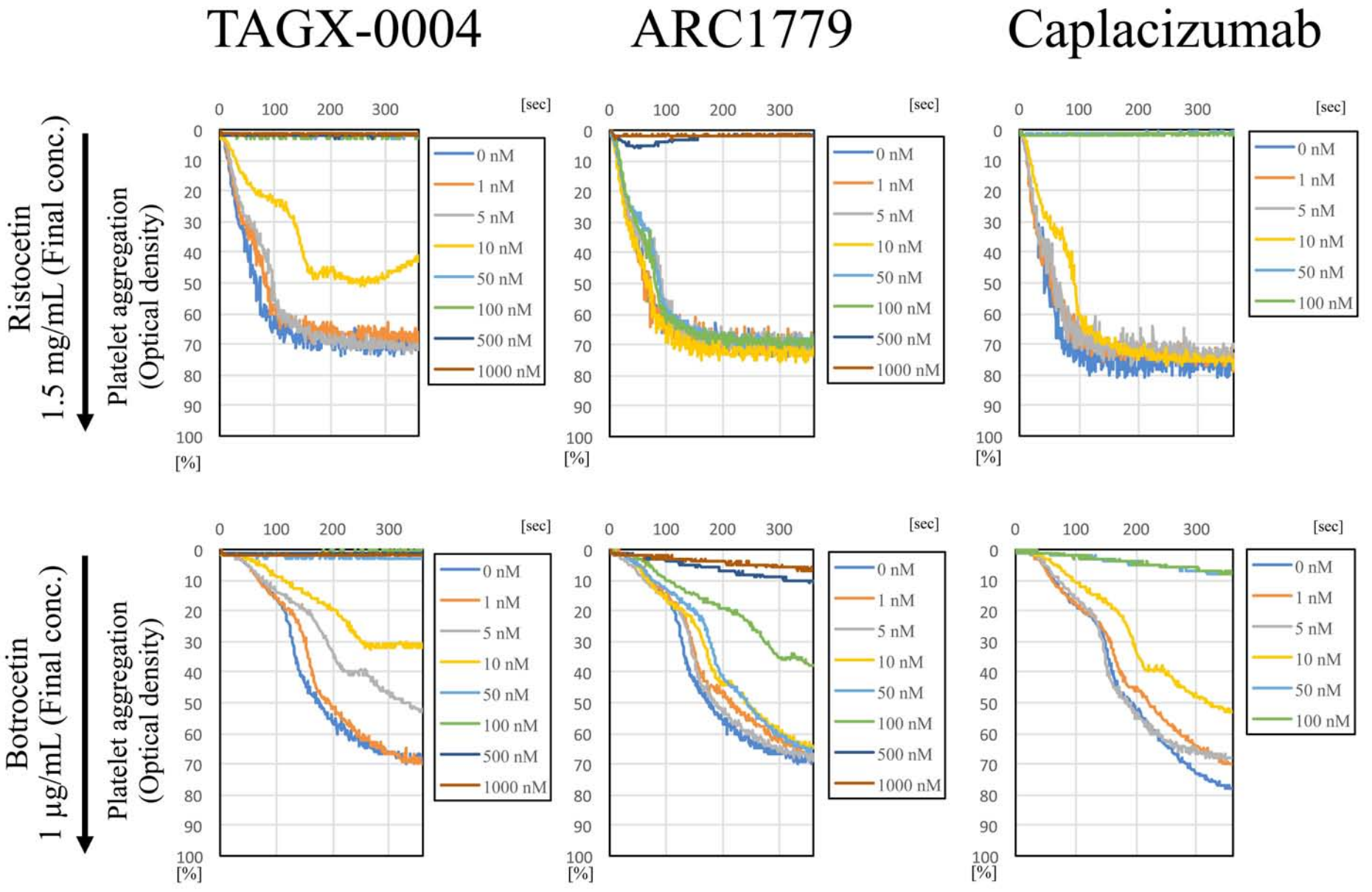
Figure 5 Analysis of caplacizumab binding sites to the VWF A1 domain using alanine-scanning mutants with surface plasmon resonance (SPR)

We performed SPR analysis to investigate caplacizumab binding sites to the VWF A1 domain. After immobilizing caplacizumab on the sensor chip CM5, 16 alanine-substituted VWF A1 mutants were analyzed. The relative binding volume of wild-type VWF A1 was defined as 100%. The binding amount of each mutant was expressed as a ratio relative to wild type. Five mutants were considered to bind to caplacizumab based on having a relative ratio above the cutoff of 80%. These results indicate that these five amino acids (K1362, R1392, R1395, R1399, and K1406) in the VWF A1 domain were important in caplacizumab binding.

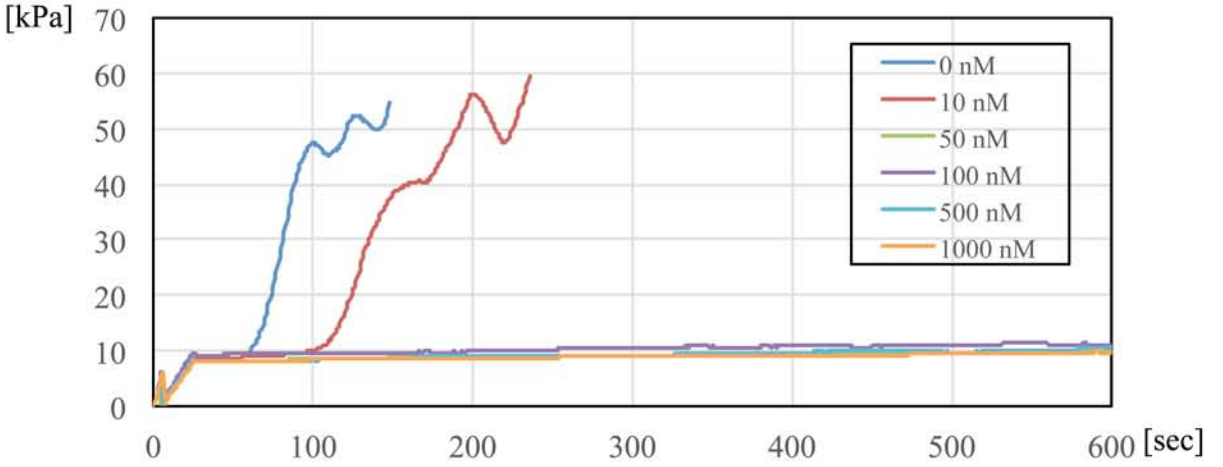
Figure 6 VWF A1 domain binding sites to TAGX-0004, ARC1779, and caplacizumab

Amino acids colored yellow did not contribute to VWF A1 binding to aptamers or caplacizumab. Amino acids colored red were necessary for binding to aptamers or caplacizumab. Three amino acids (F1366, R1395, and R1399) in the VWF A1 domain were identified as TAGX-0004 binding sites. For binding to ARC1779, five amino acids

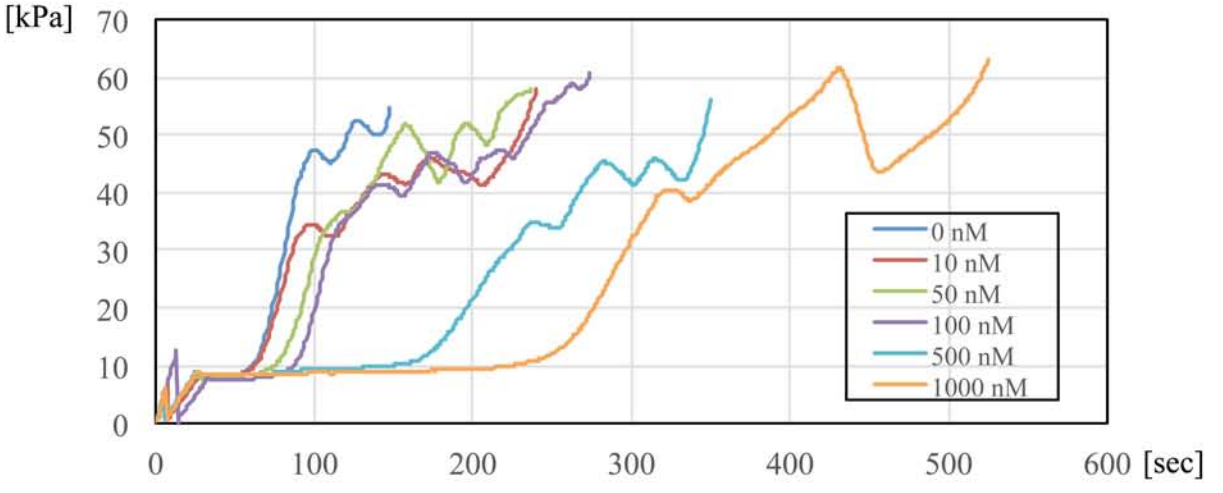
(R1287, K1362, R1392, R1395, and R1399) were important. Finally, five amino acids (K1362, R1392, R1395, R1399, and K1406) were important for binding to caplacizumab.


TAGX-0004
ARC1779
Caplacizumab
Ristocetin
1.5 mg/mL (Final conc.)
Botrocetin
1 μg/mL (Final conc.)
Platelet aggregation
(Optical density)
[%]
[sec]
0
100
200
300
0
10
20
30
40
50
60
70
80
90
100
0 nM
1 nM
5 nM
10 nM
50 nM
100 nM
500 nM
1000 nM

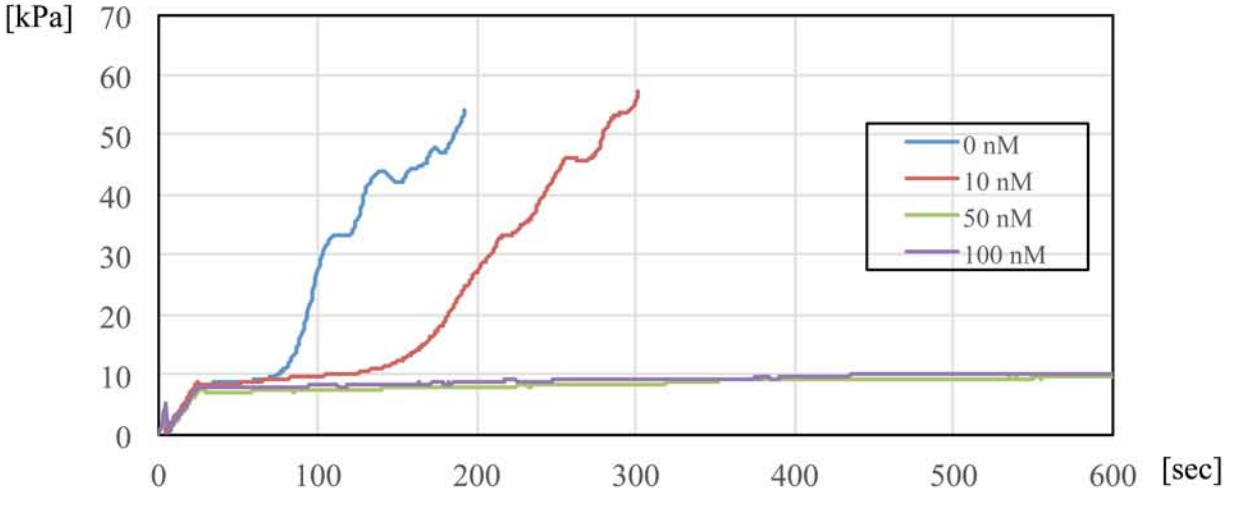
TAGX-0004

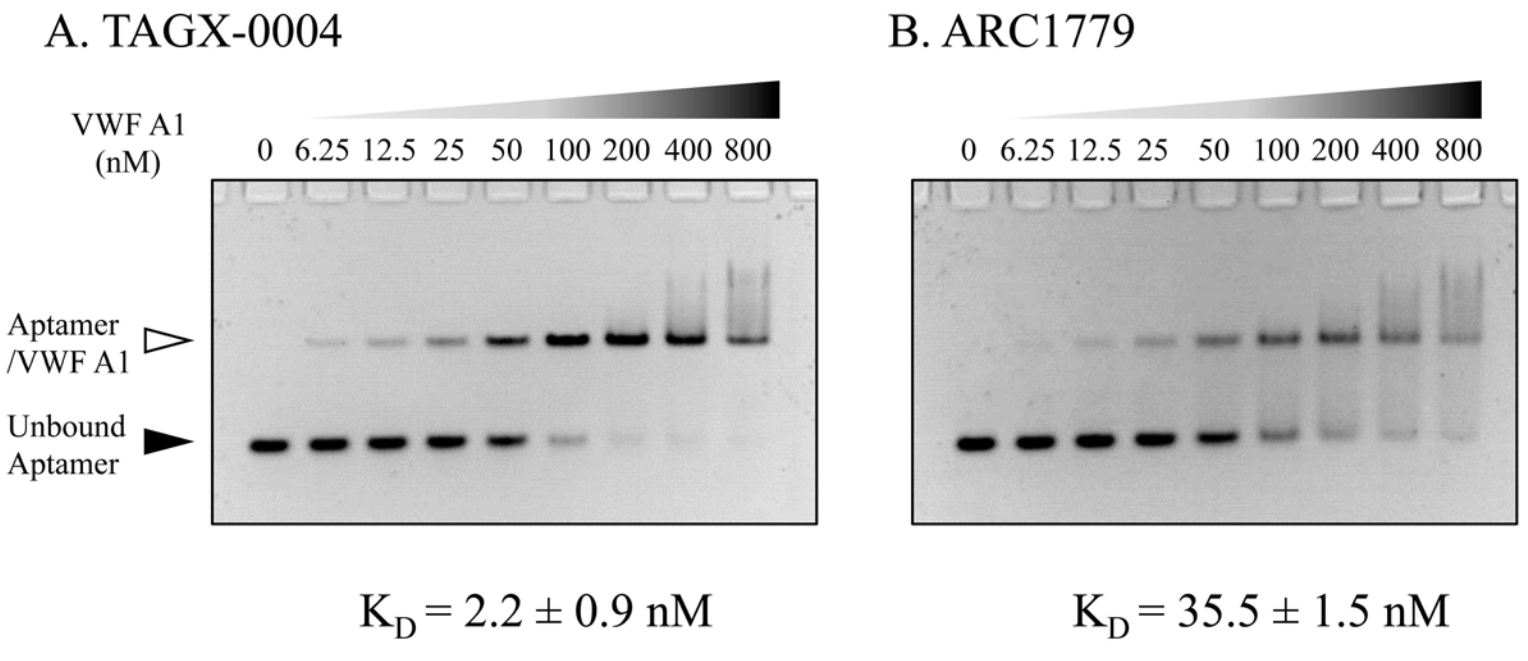


ARC1779

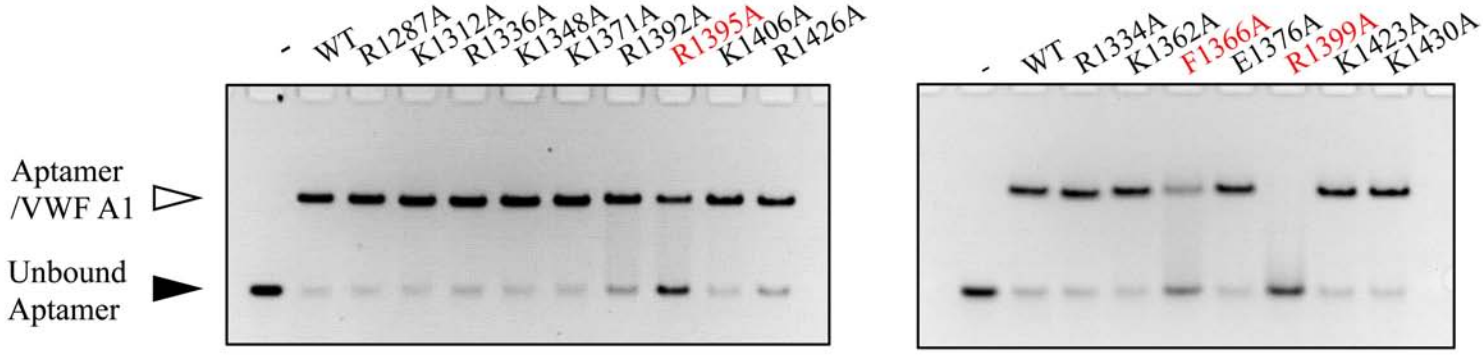


Caplacizumab

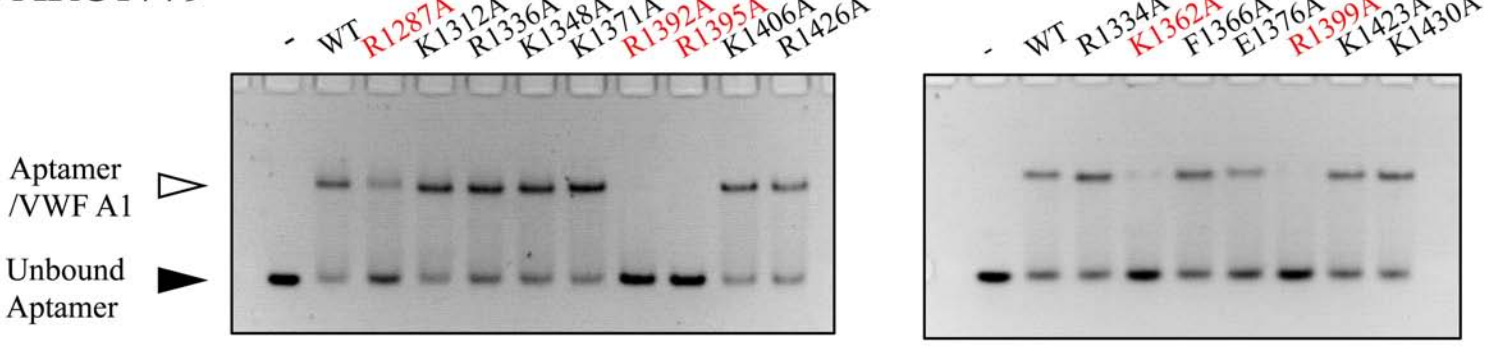


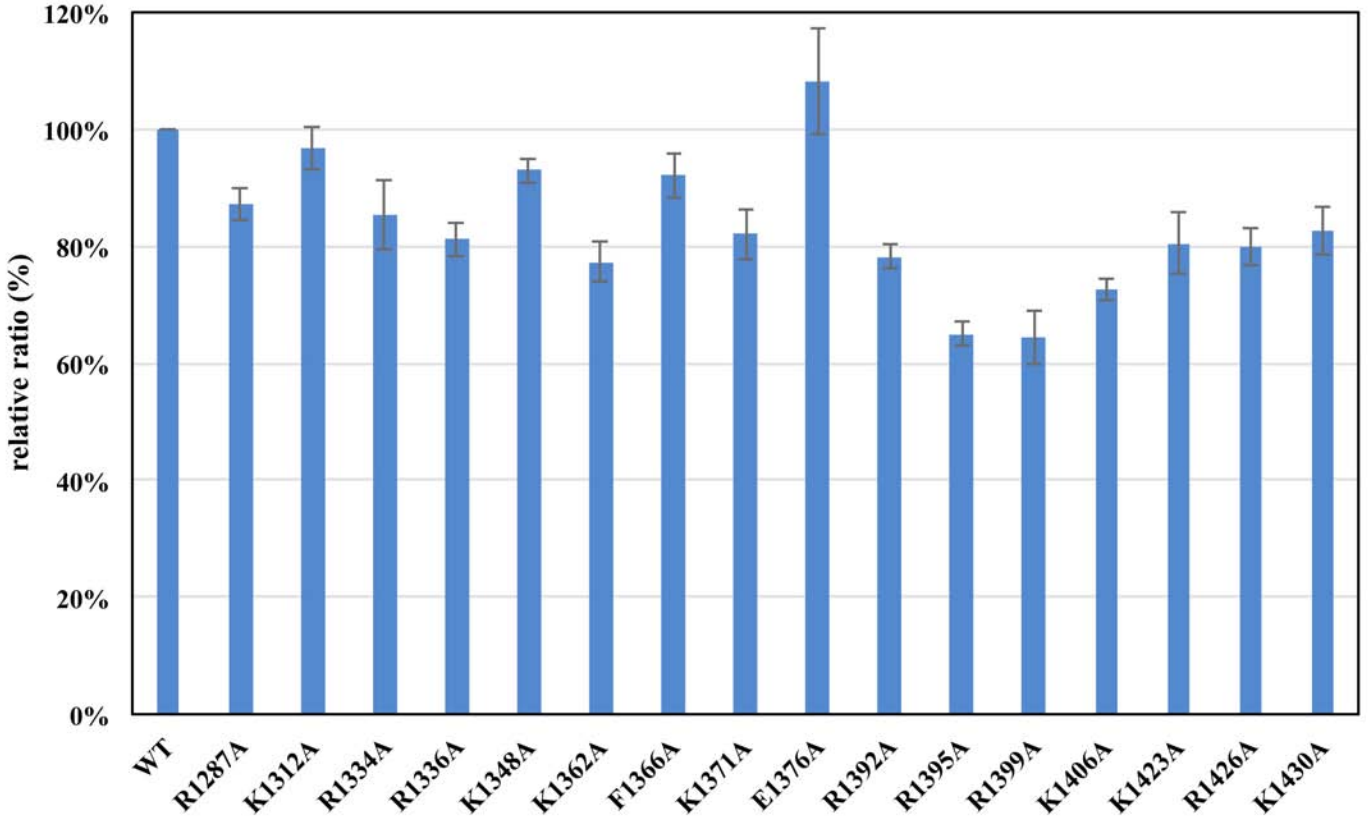

A. TAGX-0004
B. ARC1779
VWF A1
(nM)
0 6.25 12.5 25 50 100 200 400 800
0 6.25 12.5 25 50 100 200 400 800
Aptamer
/VWF A1
Unbound
Aptamer
$K_D = 2.2 \pm 0.9$ nM
$K_D = 35.5 \pm 1.5$ nM

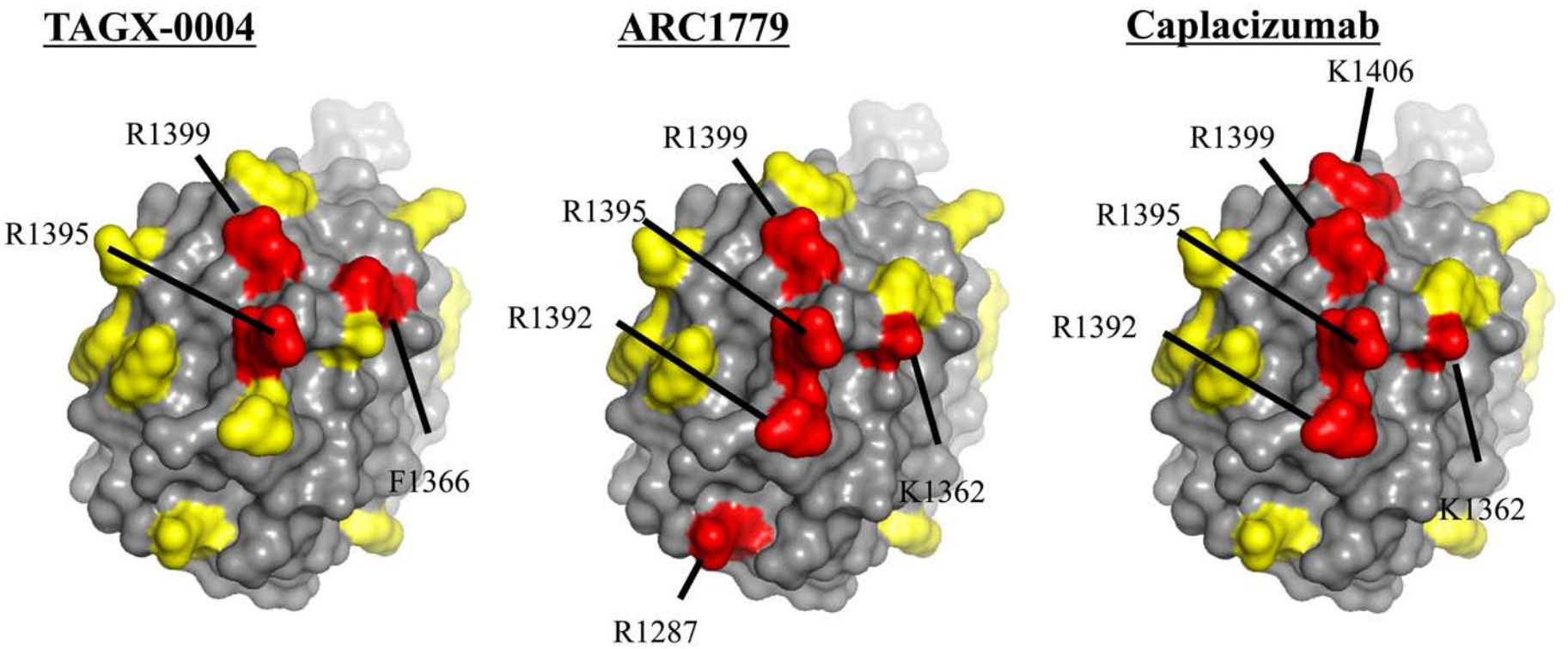
A. TAGX-0004



B. ARC1779




120%
100%
80%
60%
40%
20%
0%
relative ratio (%)
WT
R1287A
K1312A
R1334A
R1336A
K1348A
K1362A
F1366A
K1371A
E1376A
R1392A
R1395A
R1399A
K1406A
K1423A
R1426A
K1430A


TAGX-0004
R1399
R1395
F1366
ARC1779
R1399
R1395
R1392
K1362
R1287
Caplacizumab
K1406
R1399
R1395
R1392
K1362

Supplemental Information

Novel aptamer to von Willebrand factor A1 domain (TAGX-0004) shows total inhibition of thrombus formation superior to ARC1779 and comparable to caplacizumab

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Running title: Novel DNA aptamer targeting VWF A1 domain

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Supplemental Methods

Sources of nucleoside, oligonucleotides, nanobody, and protein

The artificial nucleoside triphosphate (dDsTP) was synthesized as described previously.[1, 2] The oligonucleotides TAGX-0004 and ARC1779 (without polyethylene glycol [PEG]) were synthesized by and purchased from GeneDesign, Inc. (Osaka, Japan). The oligonucleotide sequence of TAGX-0004 (as Rn-DsDs-51mh2)[3] and that of ARC1779[4] were reported previously. The anti-human VWF nanobody caplacizumab (as TAB-234) was purchased from Creative-Biolabs (NY, USA). Briefly, the nanobody was expressed in *E. coli*, purified by Ni affinity chromatography and ultrafiltration, and then subjected to 0.2-micron sterile filtration. The recombinant human VWF A1 domain protein was purchased from U-Protein Express BV (Utrecht, Netherlands), and used for the measurement of binding affinity to anti-human VWF A1 agents using SPR.

Alanine-scanning mutagenesis

Based on previous reports that described the interaction between VWF A1 and GPIb,[5] botrocetin,[6] or ARC1172,[7] we designed 16 alanine-substituted mutants of the human VWF A1 domain (R1287, K1312, R1334, R1336, K1348, K1362, F1366, K1371, E1376, R1392, R1395, R1399, K1406, K1423, R1426, and K1430). These alanine-substituted mutants were expressed the A1 domain of 217 residues, which are located V1252 to T1481. Of these, R1334, K1362, F1366, K1371, and E1376 were previously reported to bind to the GPIb domain based on the crystal structure of the GPIbα–VWF A1 complex.[5] Five amino acid residues (K1362, R1392, R1395, R1399, and K1430) were found to be important to contribute the BIPA.[6] Six amino acid residues (R1287, K1362, F1366, R1392, R1395, and K1423) were reported to be binding sites for ARC1172 (from which ARC1779 was derived).[7] These mutant proteins were generated using a cell-free expression system (Taiyo Nippon Sanso Corporation, Tokyo, Japan).

Platelet Aggregation Test (PAT)

PAT was performed with PRP313M aggregometer (TAIYO Instruments INC, Osaka, Japan). Aggregation inducing substances and their final concentration were as follows; ristocetin (1.5 mg/mL), botrocetin (1.0 μg/mL), collagen (4 μg/mL), epinephrine

(1.0×10^{-4} M) and adenosine diphosphate (ADP) (1.0×10^{-5} M). The purification method of botrocetin from the venom of *Bothrops jararaca* was previously reported in detail.[8] Ristocetin was purchased from ABP Ltd. (Middlesex, UK). Collagen, epinephrine and ADP were purchased from ARKLAY Inc. (Kyoto, Japan). The citrated blood was obtained from a healthy volunteer who had taken neither antiplatelet nor anticoagulation agents for at least 7 days. Platelet rich plasma (PRP) was prepared from the collected citrated blood by centrifugation at 60 g in 25°C for 20 min, then the remaining PRP was centrifuged at 2000 g for 20 min to obtain platelet poor plasma (PPP). Platelet count of PRP was measured in each analysis and confirmed around 300×10^{9}/L. For PAT, PRP of 230 μL was aliquoted into a cuvette, and while mixing with a stir bar, 10 μL of each agent (PBS, aptamer or caplacizumab solution) was added and incubated at 37°C for 1 min. Then, 10 μL of each aggregation inducing substance was applied to the cuvette. The final concentration of the aptamer ranged from 0 to 1000 nM, and those of caplacizumab from 0 to 100 nM. The change in light transmission of PRP at 37°C for 6 min was monitored and the aggregation rate was calculated using that of PPP as a background. The inhibitory effect was evaluated by comparing the aggregation rate of each aptamer to that of PBS.

Total thrombus formation analysis system (T-TAS)

The T-TAS® (Zacros, Fujimori Kogyo Co. Ltd., Tokyo, Japan) is a micro-chip flow-chamber device used to visually and quantitatively analyze thrombus formation in whole blood samples under various blood flow conditions.[9] A platelet chip coated with type I collagen was used to assess platelet thrombus formation at high shear rates. Whole blood anticoagulated with hirudin was obtained from a healthy volunteer. Then, 490 μL of whole blood sample and 10 μL of each agent (PBS, aptamer, or caplacizumab solution) were mixed gently. The final concentrations of the aptamers were 0, 10, 50, 100, 500, and 1000 nM, and those of caplacizumab were 0, 10, 50, and 100 nM. Subsequently, 320 μL of each blood sample was applied to the collagen-coated capillary at flow rates of 24 μL/min, corresponding to initial wall shear rates of 2000/second. In this assay, as the platelet thrombus grew, the microcapillaries gradually became occluded, resulting in an increase in flow pressure. The analysis was automatically stopped when the microcapillaries were completely occluded.

Electrophoresis mobility shift assay (EMSA)

The binding abilities of the two aptamers to the human VWF A1 domain and its alanine-substituted mutants were analyzed by EMSA. Each aptamer was diluted in 1× PBS, denatured at 95 °C for 5 min, and then cooled down slowly to 4 °C for 30 min. Each aptamer (final concentration 100 nM) was mixed with recombinant VWF A1 (final concentration 0 to 800 nM) in binding buffer (1× PBS, 0.005% [w/v] NP-40) and incubated at 37 °C for 30 min, then subjected to 8% native PAGE in 0.5× TBE buffer for 50 min at room temperature (200 V/cm). The aptamer-VWF A1 complexes were separated from the free aptamers, and the band patterns were detected by staining with SYBR Gold. The signal intensities were analyzed using ImageJ software.[10] The dissociation rate (K_D) was calculated by Scatchard plot analysis.

Surface plasmon resonance

Competition assays with the two aptamers were performed by SPR analysis using Biacore T200 (GE Healthcare UK Ltd., Little Chalfont, UK), as described previously.[3] Briefly, the experiment was performed at 37 °C in running buffer (1× PBS, 0.05% NP-40) and 50 mM NaOH for regeneration. Biotinylated TAGX-0004 was immobilized on a sensor chip SA (GE Healthcare UK Ltd.). The competing aptamer (TAGX-0004 or ARC1779, final concentration 0 to 100 nM) was mixed with VWF A1 (final concentration 5 nM) and incubated at 37 °C for 30 min, then the mixed samples were injected in running buffer. Measurement conditions used were at a flow rate of 100 μL/min, protein injection time of 150 sec, and dissociation time of 450 sec.

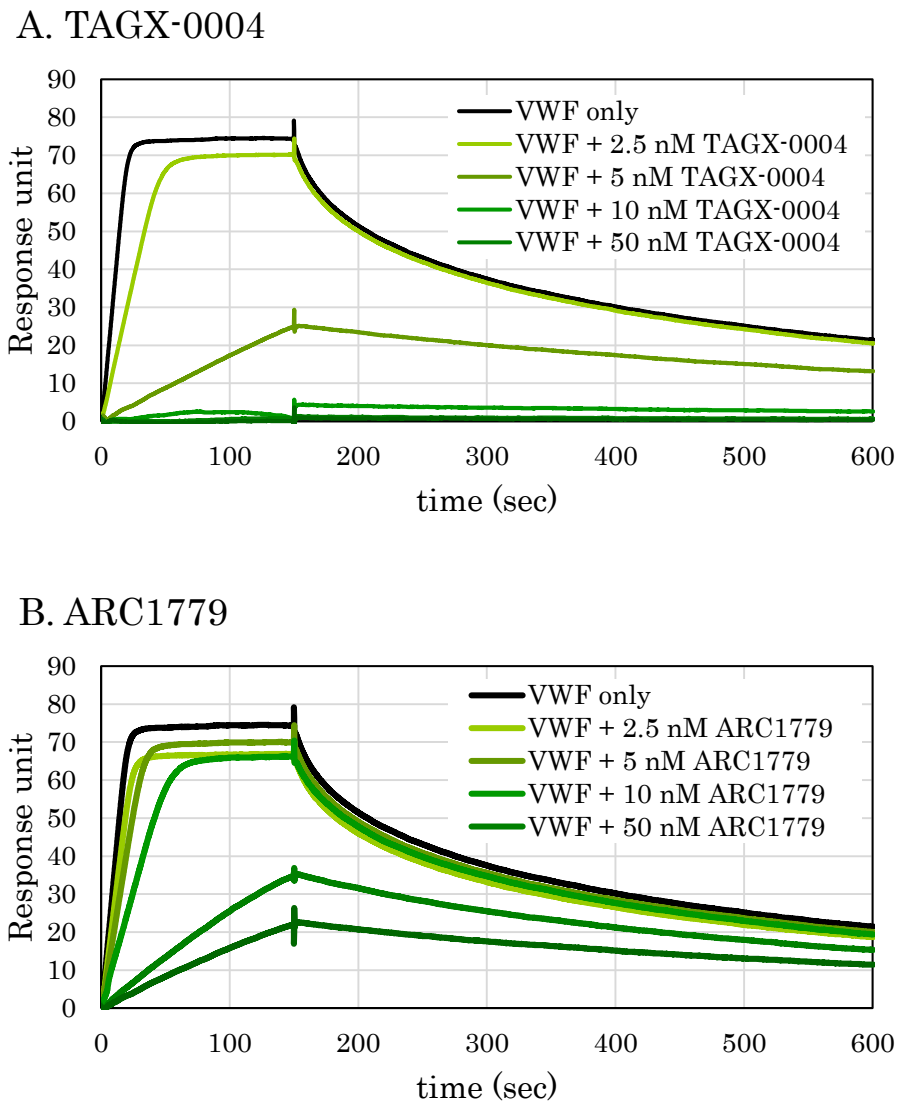
We also performed SPR analysis to investigate the binding site of the VWF A1 domain to caplacizumab. The experiment was performed at 25 °C in running buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 3 mM EDTA, 0.005% Tween20) and 10 mM glycine-HCl pH 1.5 for regeneration. Caplacizumab was immobilized on a sensor chip CM5 (GE Healthcare UK Ltd.) by amino coupling, then a series of alanine-substituted mutants of VWF A1 (final concentration 5 nM) were injected as the analytes. Measurement conditions tested were at a flow rate of 50 μL/min, contact time of 150 sec, and dissociation time of 450 sec.

Structure models of the VWF A1 domain

Three-dimensional (3D) structure models of the VWF A1 domain were visualized

with the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, USA). Using these 3D models, the amino acid residues essential for binding to TAGX-0004, ARC1779, or caplacizumab were compared each other.

Supplemental Figure



Supplementary Figure 1 Competition assay using surface plasmon resonance (SPR) to analyze the binding activity of TAGX-0004 and ARC1779

A competition assay was performed with SPR using Biacore. Biotinylated TAGX-0004 was immobilized on the sensor chip SA. Then, wild-type (WT) VWF A1 with a competitor aptamer (TAGX-0004 or ARC1779) was injected. The upper panel shows results of a self-competition assay using TAGX-0004; the amount of WT VWF A1 that bound to the chip decreased with increasing concentrations of competitor TAGX-0004. For ARC1779, the lower panel shows that the response unit value (the amount of WT VWF A1) also decreased with increasing concentrations of ARC1779. These results indicate that TAGX-0004 and ARC1779 bound to similar regions on the VWF A1 domain.

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