

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

**Kazuya Sakai,<sup>1</sup> Tatsuhiko Someya,<sup>2</sup> Kaori Harada,<sup>2</sup> Hideo Yagi,<sup>1</sup> Tai Matsui<sup>3</sup> and Masanori Matsumoto<sup>1</sup>**

<sup>1</sup>Department of Blood Transfusion Medicine, Nara Medical University, Kashihara and <sup>2</sup>TAGCyx Biotechnologies Inc, Tokyo and <sup>3</sup>Clinical Laboratory Medicine, Fujita Health University School of Medical Sciences, Toyoake, Japan

©2020 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2019.235549

Received: August 16, 2019.

Accepted: December 18, 2019.

Pre-published: December 19, 2019.

Correspondence: *MASANORI MATSUMOTO* - mmatsumo@naramed-u.ac.jp

---

## 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**

Kazuya Sakai,<sup>1</sup> Tatsuhiko Someya,<sup>2</sup> Kaori Harada<sup>2</sup>, Hideo Yagi,<sup>1</sup> Tai Matsui,<sup>3</sup> and Masanori Matsumoto<sup>1</sup>

<sup>1</sup>Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Japan;

<sup>2</sup>TAGCyx Biotechnologies Inc, Tokyo, Japan

<sup>3</sup>Clinical Laboratory Medicine, Fujita Health University School of Medical Sciences, Toyoake, Japan

Running title: Novel DNA aptamer targeting VWF A1 domain

Corresponding author:

Masanori Matsumoto, MD, PhD

Professor of Department of Blood Transfusion Medicine

Nara Medical University

840 Shijyo-cho, Kashihara city, Nara

634-8522, Japan

## Supplemental Methods

### *Sources of nucleoside, oligonucleotides, nanobody, and protein*

The artificial nucleoside triphosphate (dDsTP) was synthesized as described previously.<sup>1, 2</sup> 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)<sup>3</sup> and that of ARC1779<sup>4</sup> 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,<sup>5</sup> botrocetin,<sup>6</sup> or ARC1172,<sup>7</sup> 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 GPIb $\alpha$ -VWF A1 complex.<sup>5</sup> Five amino acid residues (K1362, R1392, R1395, R1399, and K1430) were found to be important to contribute the BIPA.<sup>6</sup> Six amino acid residues (R1287, K1362, F1366, R1392, R1395, and K1423) were reported to be binding sites for ARC1172 (from which ARC1779 was derived).<sup>7</sup> 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  $\mu$ g/mL), collagen (4  $\mu$ g/mL), epinephrine

( $1.0 \times 10^{-4}$  M) and adenosine diphosphate (ADP) ( $1.0 \times 10^{-5}$  M). The purification method of botrocetin from the venom of *Bothrops jararaca* was previously reported in detail.<sup>8</sup> 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  $300 \times 10^9$ /L. For PAT, PRP of 230  $\mu$ L was aliquoted into a cuvette, and while mixing with a stir bar, 10  $\mu$ L of each agent (PBS, aptamer or caplacizumab solution) was added and incubated at 37°C for 1 min. Then, 10  $\mu$ L 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<sup>®</sup> (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.<sup>9</sup> 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  $\mu$ L of whole blood sample and 10  $\mu$ L 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  $\mu$ L of each blood sample was applied to the collagen-coated capillary at flow rates of 24  $\mu$ L/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.<sup>10</sup> 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.<sup>3</sup> 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  $\mu$ L/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  $\mu$ L/min, contact time of 150 sec, and dissociation time of 450 sec.

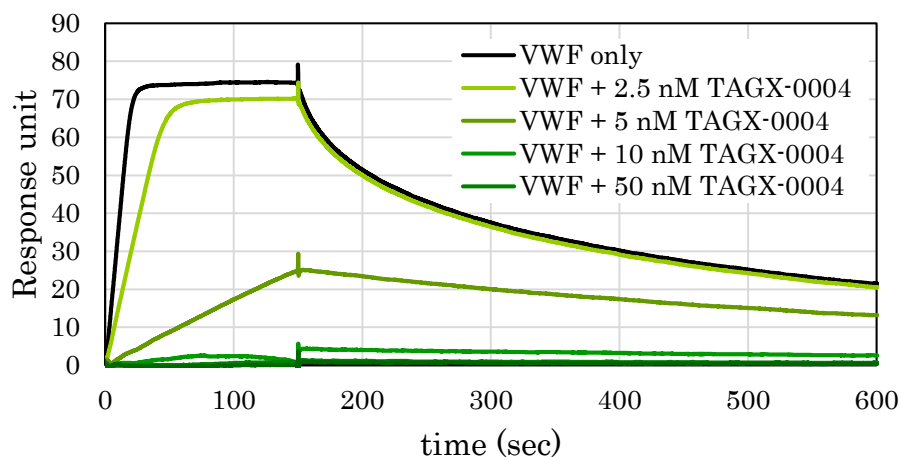
### *Structure models of the VWF A1 domain*

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

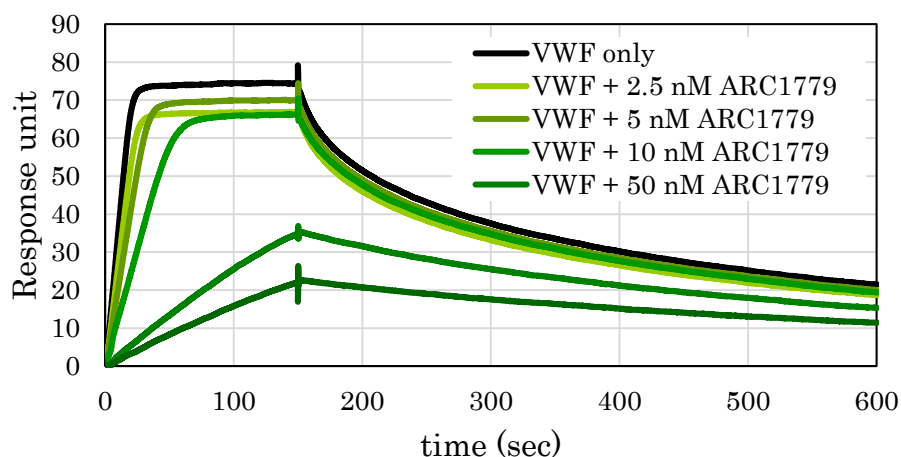
with the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, USA). Using these 3D models, the amino acid residues essential for binding to TAGX-0004, ARC1779, or caplacizumab were compared each other.

## Supplemental Figure

### A. TAGX-0004



### B. ARC1779



### 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.

## Supplemental References

1. Hirao I, Kimoto M, Mitsui T, et al. An unnatural hydrophobic base pair system: site-specific incorporation of nucleotide analogs into DNA and RNA. *Nat Methods*. 2006;3(9):729-735.
2. Yamashige R, Kimoto M, Takezawa Y, et al. Highly specific unnatural base pair systems as a third base pair for PCR amplification. *Nucleic Acids Res*. 2012;40(6):2793-2806.
3. Matsunaga KI, Kimoto M, Hirao I. High-Affinity DNA Aptamer Generation Targeting von Willebrand Factor A1-Domain by Genetic Alphabet Expansion for Systematic Evolution of Ligands by Exponential Enrichment Using Two Types of Libraries Composed of Five Different Bases. *J Am Chem Soc*. 2017;139(1):324-334.
4. Diener JL, Daniel Lagasse HA, Duerschmied D, et al. Inhibition of von Willebrand factor-mediated platelet activation and thrombosis by the anti-von Willebrand factor A1-domain aptamer ARC1779. *J Thromb Haemost*. 2009;7(7):1155-1162.
5. Huizinga EG, Tsuji S, Romijn RA, et al. Structures of glycoprotein Ibalph and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-1179.
6. Matsushita T, Meyer D, Sadler JE. Localization of von willebrand factor-binding sites for platelet glycoprotein Ib and botrocetin by charged-to-alanine scanning mutagenesis. *J Biol Chem*. 2000;275(15):11044-11049.
7. Huang RH, Fremont DH, Diener JL, Schaub RG, Sadler JE. A structural explanation for the antithrombotic activity of ARC1172, a DNA aptamer that binds von Willebrand factor domain A1. *Structure*. 2009;17(11):1476-1484.
8. Fujimura Y, Titani K, Usami Y, et al. Isolation and chemical characterization of two structurally and functionally distinct forms of botrocetin, the platelet coagglutinin isolated from the venom of *Bothrops jararaca*. *Biochemistry*. 1991;30(7):1957-1964.
9. Hosokawa K, Ohnishi T, Kondo T, et al. A novel automated microchip flow-chamber system to quantitatively evaluate thrombus formation and antithrombotic agents under blood flow conditions. *J Thromb Haemost*. 2011;9(10):2029-2037.
10. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9(7):671-675.