Supplementary information for:

Inhibition of thrombin activity by a covalent-binding aptamer and reversal by the complementary strand antidote

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1. General

All the reagents and solvents were purchased commercially and used without further purification. Native TBA, TBA with introduction of a relatively long spacer and a terminal alkyne replacing a thymine residue (T_x), complementary strand (CS) antidote, and carboxyfluorescein (FAM)-tagged CS were synthesized by Integrated DNA Technologies (IDT) Inc. (Table S1).

Table S1. Nucleic acids purchased from IDT.

sample	sequence
native TBA	5′-GGTTGGTGTGGTTGG-3′
alkyne-containing TBA (T_3)	5′-GGXTGGTGTGGTTGG-3′
alkyne-containing TBA (T_9)	5′-GGTTGGTGXGGTTGG-3′
alkyne-containing TBA (T_{12})	5′-GGTTGGTGTGGXTGG-3′
CS antidote	5′-CCAACCACACCAACC-3′
FAM-tagged CS	FAM-5′-CCAACCACACCAACC-3′

Human α-thrombin (Haematologic Tech Inc, # HCT-0020, USA) and fibrinogen from human plasma (Aldrich, #9001-32-5, USA) were purchased commercially.

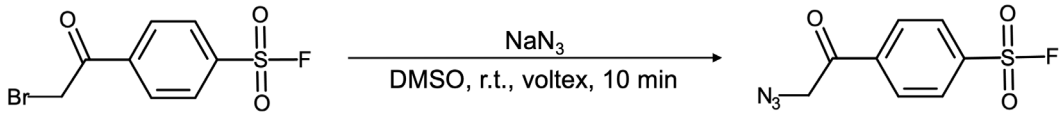
NMR experiments were performed at 25 ºC using a 500 MHz spectrometer (JNM-ECA500, Jeol Resonance, Japan). Liquid chromatography (LC) analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, USA) using a 0–100% gradient of acetonitrile containing 0.1% formic acid at a flow rate of 300 μL per minutes, equipped with a C18 reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific, USA) connected to a photodiode array (PDA) and/or a LCQ-Fleet ion trap mass spectrometer. A small scale quantitative analysis of aptamers was carried out by using a reversed-phase semi-micro HPLC system (PU-2085 with C18 column, JASCO, Japan) connected to a fluorescence detector followed by a PDA. The aptamers were separated using a 0–60% gradient of acetonitrile containing 20 mM triethylamine acetate aqueous solution (pH 7.4) for 26 minutes at a flow rate of 200 μL per minutes. Electrospray ionization-time-of-flight-mass spectrometry (ESI-TOF-MS; JMS-T100 AccuTOF, Jeol Resonance, Japan) was performed by dissolving the analyte in methanol and directly injecting into the instrument. The trypsinized peptides were

analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS, UltrafleXtreme, Bruker, USA) with several matrices (*e.g.*, 2,5-dihydroxybenzoic acid, 3-amino-4-hydroxybenzoic acid).

All images of stained gel and in-gel fluorescence were captured by ChemDoc XRS+ (BioRad Laboratories Inc., USA) and band intensities were quantified using Image Lab software (BioRad Laboratories Inc., USA).

2. Synthesis of covalent-binding TBA

2.1. Synthesis of warhead; **1**



The warhead **1** was synthesized on a preparative scale according to the following procedure. 4-(2-bromoacetyl)-benzene-1-sulfonyl fluoride (71 μmol, Aldrich #00364, USA) and sodium azide (65 μmol, WAKO #195-11092, Japan) were mixed in 0.32 mL of dimethyl sulfoxide (DMSO). The reaction mixture was vortexed for 10 minutes at room temperature, then mixed with cold water (0.5 mL) and extracted with ethyl acetate (1 mL). The collected organic phase was washed with saturated $NaHCO_3$ (0.5 mL × 2) and brain (0.5 mL × 2), dried over Na_2SO_4, and evaporated to yield a yellow solid pure product (12 mg, yield 49%). Confirmation of the purified warhead **1** was performed by ^{1}H, ^{13}C, and ^{19}F NMR (Fig. S2).

1: ^{1}H NMR ($CDCl_3$, 500 MHz,) δ 8.15 (m, 4H), 8.46 (s, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 192.04, 139.62, 137.60, 129.19, 55.37; ^{19}F NMR ($CDCl_3$, 470 MHz) δ 65.95.

2.2. Synthesis of warhead-conjugated TBA via CuAAC reaction

Tris (3-hydroxypropyltriazolylmethyl) amine (in water, 0.50 μmol, Aldrich # 762342, USA) and copper (II) sulfate (in water, 0.25 μmol, Aldrich # 451657, USA) were mixed. Then, each alkyne-containing TBA (in water, 10 nmol) and warhead (in DMSO, 0.50 μmol) was added, and the mixture was reacted for 1 hour at room temperature after addition of ascorbic acid (in water, 0.40 μmol, Aldrich # A92902, USA). The crude reaction product was purified by ethanol precipitation. To the crude product, sodium acetate (in water, 9 μmol) and -20 ºC cooled ethanol were added and incubated at -20 ºC for 1hour. After centrifugation (15000 rpm, 20 minutes, 4 ºC), the supernatant was removed, and the pellet washed with 70% ethanol. The residue was

dissolved in nuclease-free water and the purified warhead-conjugated TBA was identified by LC analysis (Fig. S3). We also tried to identify the warhead-conjugated TBA by MS analysis (*e.g.*, ESI-TOF/MS, MALDI-TOF/MS, LC-MS). However, we could not observe the expected *m/z* value of the warhead-conjugated TBA both in positive and negative ionization modes, likely due to the low ionization efficiency of the fluorine derivative and/or due to the divergent ionization-mode requirements of the triazole- and nucleic acid-parts off the molecule further contributing to the ionization difficulty.

2.3. Optimization of warhead position

Native TBA and each warhead-conjugated TBA (i.e., TBA_3, TBA_{12}, TBA_9) (0.33 mM) were mixed with thrombin (25 μM) in Dulbecco's phosphate-buffered saline (D-PBS), and incubated for 12 hours at 37 ºC. The samples were mixed with SDS-PAGE Sample Buffer (Wako) and separated by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Whole proteins were visualized by Coomassie Brilliant Blue (CBB) staining.

3. Evaluation of TBA_3 covalent-binding

3.1. TBA_3-concentration dependent mobility shift of thrombin on gel electrophoresis

Various molar concentrations of TBA_3 and a constant molar concentration of thrombin (25 μM) were mixed in D-PBS and incubated for 12 hours at 37 ºC. After addition of the sample buffer and separation by 13% SDS-PAGE, proteins were visualized by CBB staining.

3.2. Time-dependent change of covalent-binding efficiency

For assessment of time-dependent bond formation of TBA_3, we mixed TBA_3 (100 μM) and thrombin (25 μM) with various incubate time in D-PBS at 37 ºC. It was mixed with the sample buffer, separated by 13% SDS-PAGE. Whole proteins were visualized CBB staining. The unreacted-thrombin band was quantified by Image Lab software and the density of unreacted-thrombin band at 0 minute was normalized to 100% and the relative density at each time was quantified.

3.3. Concentration-dependent change of covalent-binding efficiency

For assessment of concentration-dependent change of covalent-binding efficiency, we mixed various molar concentrations of TBA_3 and constant molar

concentrations of thrombin (25 μM) in D-PBS and incubated for 3 hours at 37 ºC. It was mixed with the sample buffer, separated by 13% SDS-PAGE. Whole proteins were visualized by CBB staining. The unreacted-thrombin band was quantified by Image Lab software and the density of unreacted-thrombin band in 0 molar of TBA_3 was normalized to 100% and the relative density of each molar concentration was quantified.

3.4 Trypsin digestion and analysis by LC-MS/MS

For LC-MS/MS analysis, TBA_3 (0.10 mM) was mixed with thrombin (25 μM) in D-PBS and incubated for 3 hours at 37 ºC. After addition of a sample buffer and separation by 13% SDS-PAGE, whole proteins were visualized by CBB staining. The stained protein bands were excised from the gel, reduced with 25 mM dithiothreitol at 65 ºC for 10 minutes, and alkylated with 55 mM iodoacetamide at room temperature for 1 hour in the dark. Digestion was carried out with modified trypsin (Promega # V5111, USA) with 25 mM n-Octyl-beta-D-thioglucoside (WAKO # 349-05361, Japan) for 12 hours at 37 ºC. The resulting peptides were analyzed using LC-MS/PDA system. The trypsinized peptides were separated using a 0-50% gradient of acetonitrile containing 0.1% formic acid during 55 minutes at a flow rate of 300 μL per minutes, and the eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode and externally calibrated. Survey MS scans were acquired in the 200-2000 *m/z* ranges. Multiply charged ions of high intensity per scan were fragmented with collision-induced dissociation in the ion trap. A dynamic exclusion window was applied within 30 seconds. All tandem mass spectra were collected using normalized collision energy of 40%. Data were acquired and analyzed with Xcalibur software v.2.07 (Thermo Scientific, USA). We also tried to analyze the trypsinized peptides by MALDI-TOF/MS, however, we could not observe ionization of the peptide-TBA_3 complex.

4. Evaluation of thrombin inhibition activity

4.1. Monitor of thrombin inhibition activity by turbidimetric assay

We mixed various molar concentrations of native TBA or TBA_3 and constant molar concentrations of thrombin (25 μM) in D-PBS and incubated for 3 hours at 37 ºC. Then, each reaction mixture was added into fibrinogen solution (in D-PBS) to give a final concentration of 2.5 nM thrombin and 1 mg/mL fibrinogen, the maximum absorbance of polymerized fibrin (288 nm) was measured every 10 seconds by

NanoPhotometer (Implen, German) using 10-mm plastic cell. In each experiment, the maximum absorbance of polymerized fibrin at 0 second was normalized to 0, and the relative absorbance of each second was quantified.

4.2. Monitor of thrombin inhibition activity by clotting time change

Various molar concentrations of native TBA or TBA_3 and a constant molar concentration of thrombin (25 μM) in D-PBS were incubated for 3 hours at 37 ºC. Each reaction mixture was supplemented with a fibrinogen solution (in D-PBS) to give a final concentration of 13 nM thrombin and 2 mg/mL fibrinogen[1], and the clotting time was measured by a BFT II Analyzer (Siemens Healthineers, Germany).

5. Evaluation of on-demand reversal property

5.1. Double strand formation between TBA and CS antidote

Thrombin (25 μM) with or without TBA_3 (100 μM) in D-PBS were incubated for 3 hours at 37 ºC. The mixture was supplemented with or without FAM-tagged CS (400 μM) for 30 minutes at 37 ºC, mixed with the sample buffer, separated by 13% SDS-PAGE, followed by an in-gel fluorescence imaging. Specificity of covalent-binding between TBA_3 and thrombin was examined, as above, but in the presence of 40% (v/v) human serum (Aldrich #H4522, USA). Whole proteins were visualized by CBB staining.

5.2. Evaluation of the reversal of thrombin inhibition by turbidimetric assay

Native TBA (3.5 nM) or TBA_3 (1.2 nM) and a constant molar concentration of thrombin (25 μM) in D-PBS was incubated for 3 hours at 37 ºC. To each reaction mixture, various molar concentration of the CS antidote was added and incubated for 30 minutes at 37 ºC. Fibrinogen solution (in D-PBS) was added to each reaction mixture to give a final concentration of 2.5 nM thrombin and 1 mg/mL fibrinogen and absorbance (288 nm) was measured every 10 seconds by NanoPhotometer (Implen, German) using a 10-mm plastic cell. Time dependent absorbance values were plotted with baseline absorbance at 0 second taken as zero.

5.3. Evaluation of the reversal of thrombin inhibition by clotting time

Native TBA (3.5 nM) or TBA_3 (1.2 nM) and thrombin (25 μM) in D-PBS were incubated for 3 hours at 37 ºC. CS antidote (5.3 or 21 nM) was added and further incubated for 30 minutes at 37 ºC. Fibrinogen solution (in D-PBS) to give a final

concentration of 13 nM thrombin and 2 mg/mL fibrinogen was added and the clotting time measured by the BFT II Analyzer.

6. Molecular docking simulations of the TBA_3 to thrombin

TBA_3 was created in Avogadro[2] (version 1.2.0) from native TBA (PDB ID = 1HAO). Docking of TBA_3 to thrombin (PDB ID = 1HAO) was performed with Moldesk Basic (version 1.1.45, Imsbio Inc., Japan) graphical user interface (GUI) of several myPresto programs[3]. Docking model (*i.e.,* globally minimized structure) of TBA_3 on thrombin was calculated by the Cosgene program of myPresto (version 5.000) using the universal force field (UFF).

7. Statistical calculations

Values are presented as mean ± S.E.M. statistical significance of differences between groups was estimated by a paired t-test. A P-value < 0.05 was considered statistically significant. Analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., USA).

8. Supplementary figures

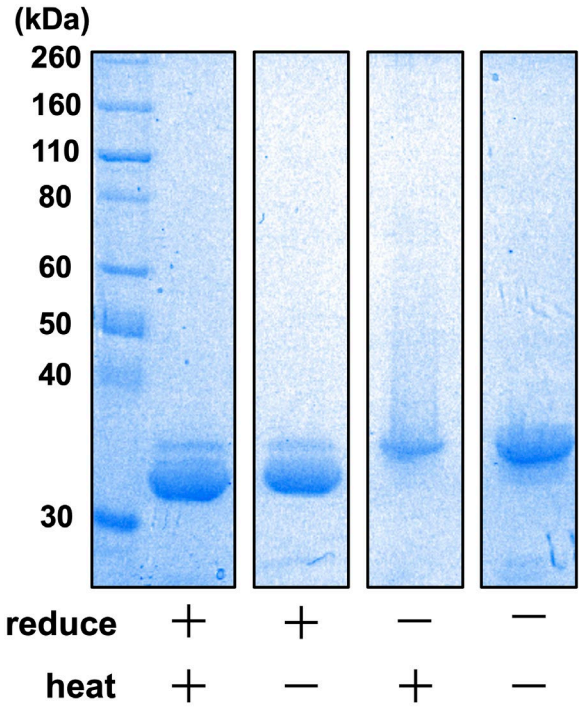


Fig. S1 *Optimization of sample preparation for SDS-PAGE.* Thrombin was prepared with or without boiling or reducing, separated by SDS-PAGE followed by CBB staining. A single thrombin band was seen without heating or reducing. We chose this simplest low-temperature and non-reducing condition for the sample preparation because it minimized potential artifacts from promiscuous covalent reaction of the warhead to the target protein.

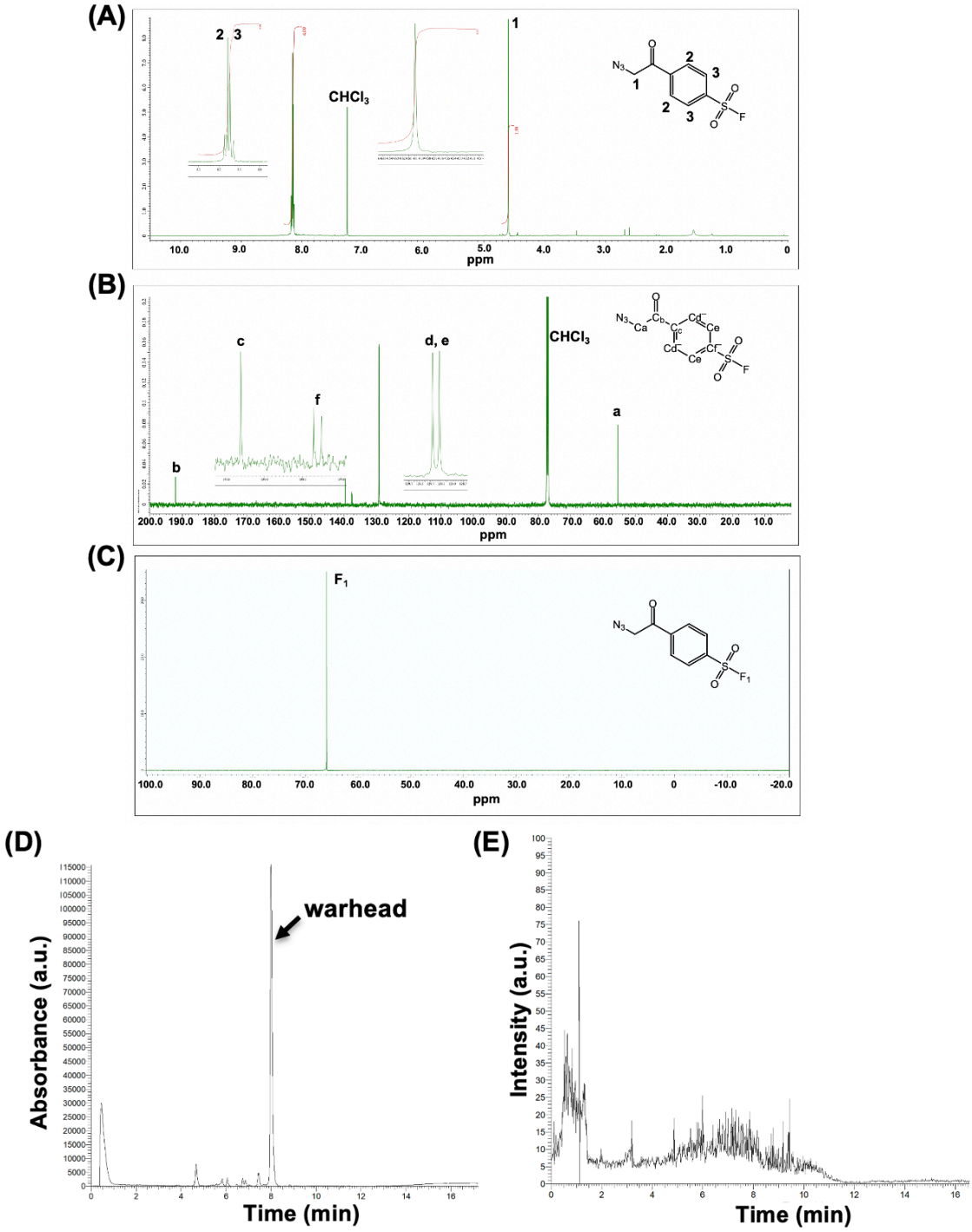


Fig. S2 *Identification of the purified warhead.* (A) ^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) ^{19}F NMR spectrum. (D) LC-absorbance (240 - 260 nm) profile of the purified warhead. (E) LC-MS total ion profile. We could not observe an expected *m/z* value of the purified warhead because of the low ionization efficiency of the fluorine derivative both in positive and negative ionization modes.

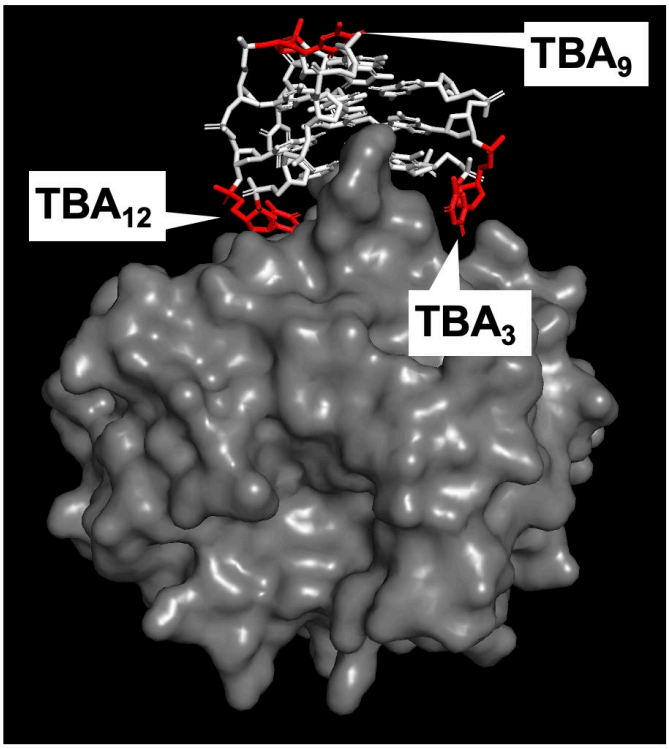


Fig. S3 *The location of each designed thymine residue visualized in PyMOL.* TBA (white), thrombin (gray surface) (PDB: 1HAO), and each designated thymine residue (red).

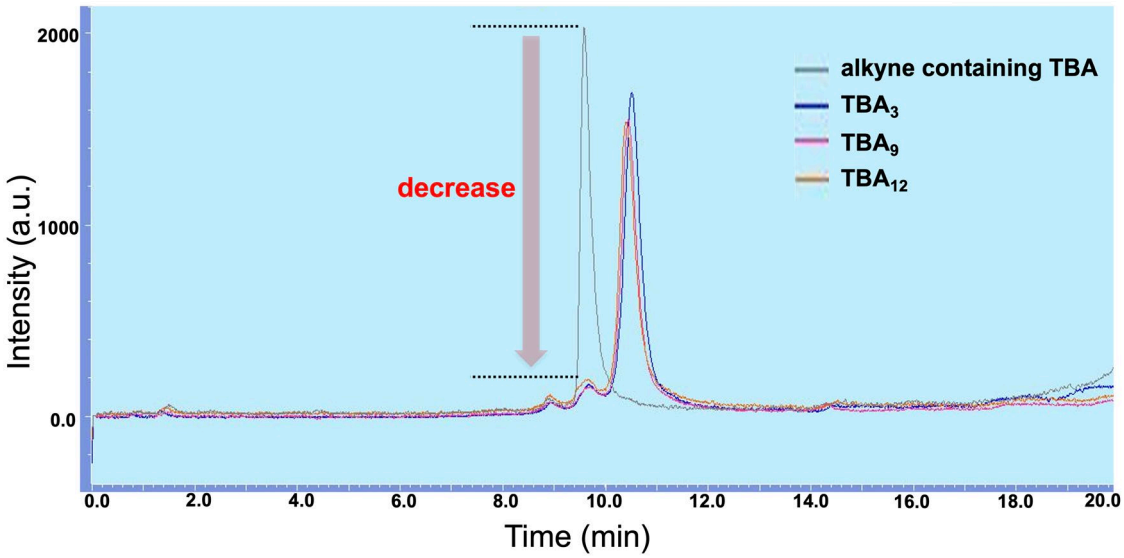


Fig. S4 *LC profiles of the crude CuAAC-reaction mixture of each alkyne-containing TBA isomer and the warhead.* (A) TBA_3 (blue line), (B) TBA_{12} (yellow line), and (C) TBA_9 (pink line), respectively, were monitored using absorbance above 270 nm. Peak areas of unreacted alkyne-containing TBAs were compared with alkyne-containing TBA before CuAAC-reaction (gray line) to estimate the conjugation reaction yields. Reaction yields were estimated about 95% from the peak area ratios.

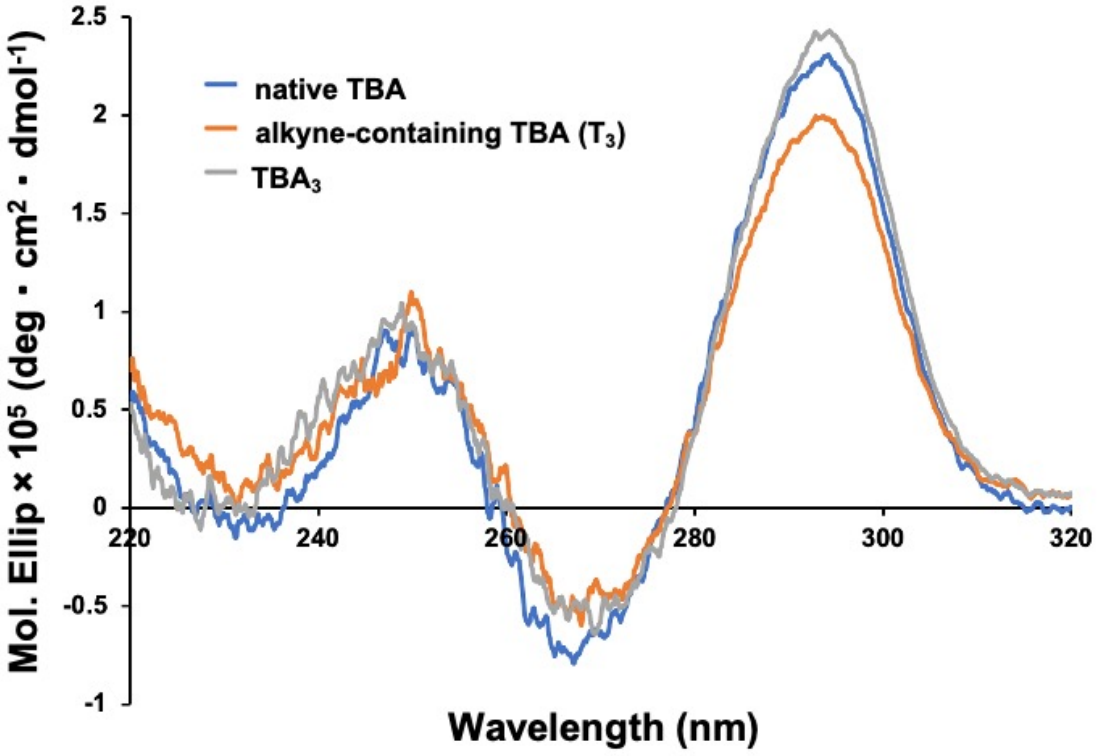


Fig. S5 *CD spectra of native TBA, alkyne-containing TBA (T_3), and TBA_3.* Native TBA, alkyne-containing TBA (T_3), or TBA_3 at 10 μM in D-PBS held at 37 ºC and spectrum scanned between 220 to 340 nm at 100 nm per minutes (Jasco Corporation J-720W Circular Dichroism Spectrometer). Triplicate measurements were averaged for the plot. The CD spectrum of the native TBA was consistent with a previous report[4] indicating that a G-quadruplex structure was present in the solution state. The introduction of an alkyne linker or a warhead did not alter the gross 3D structure of TBA.

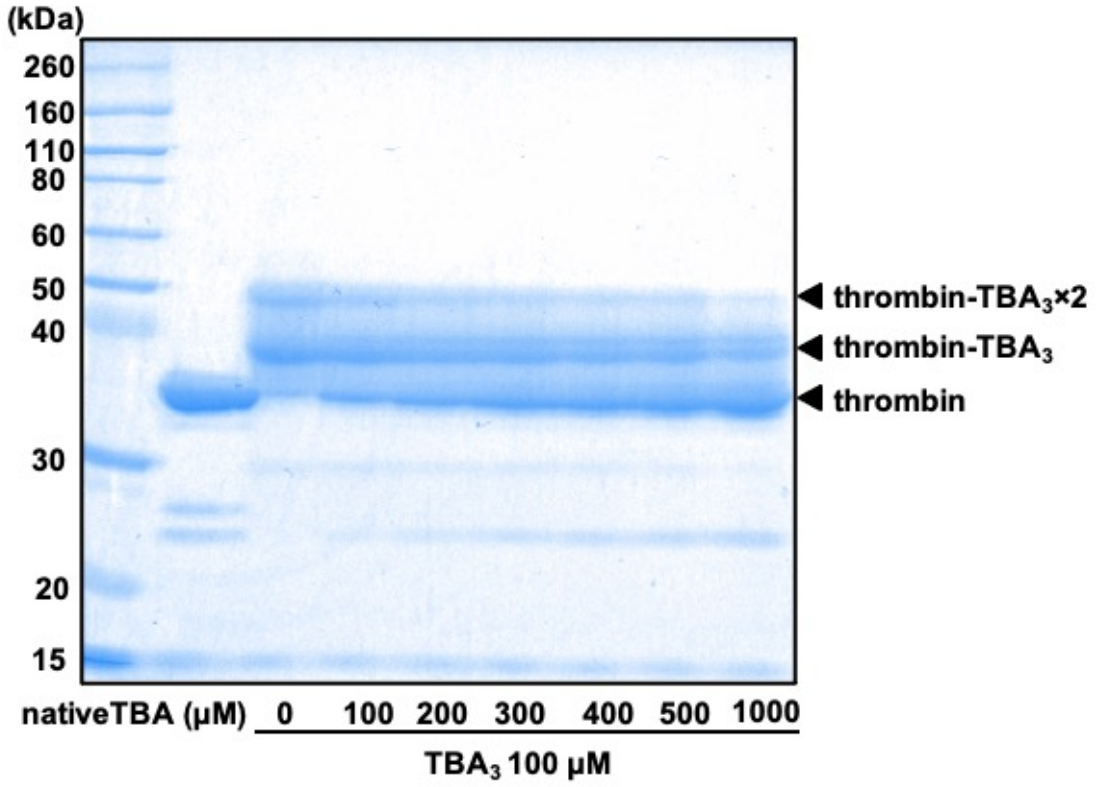


Fig. S6 *Determine of TBA3 binding site of mono- and bis-adducts by competition assay with native TBA.* The indicated amounts of native TBA were pre-incubated with thrombin (25 μM) in D-PBS for 10 minutes at room temperature. After addition of TBA_3 (100 μM), the mixture was incubated for an additional 3 hours at 37 ºC. The covalent modification of thrombin was monitored by SDS-PAGE followed by CBB staining.

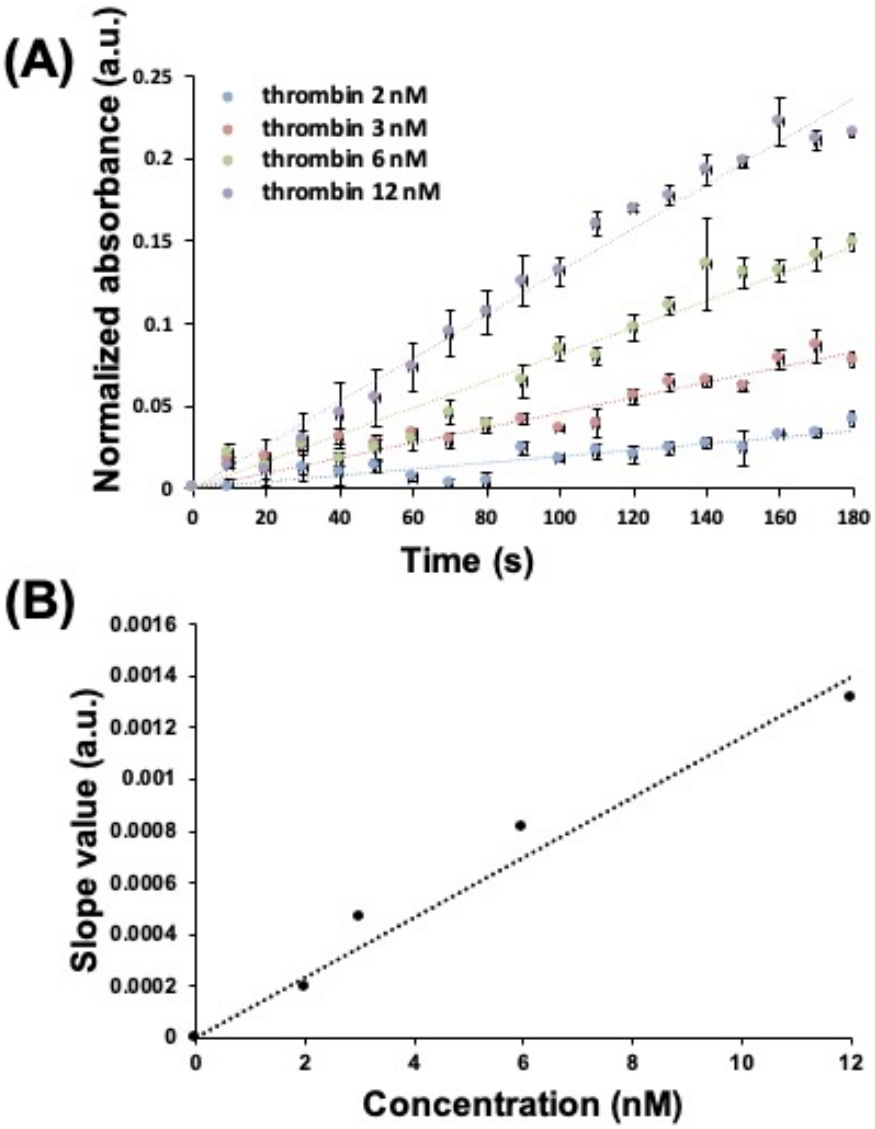


Fig. S7 *Standard curve of thrombin-activity.* Thrombin-activity was monitored by the turbidimetric assay. (A) Time-dependent change in absorbance for different thrombin concentrations. The baseline absorbance (288 nm) at 0 second was subtracted and the time dependent absorbance values plotted and a line fit by linear regression. (B) The slope values were plotted against concentration to yield the standard curve of thrombin activity as determined by this assay. The slope value (*i.e.,* thrombin-activity) was linearly correlated with thrombin concentration within the range examined.

9. References

1. Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J., Selection of Single-Stranded-DNA Molecules That Bind and Inhibit Human Thrombin. *Nature* **1992,** *355* (6360), 564-566.

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