Electronic Supplementary Information

In-situ condensation of an anti-cancer drug into fibrin gel enabling effective inhibition of tumor cell growth

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1. General

UV spectra were measured with a UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Fluorescence polarization assays were performed using an LS-55 spectrofluorophotometer (Perkin Elmer Japan Co., Ltd., Kanagawa, Japan). Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis was performed using a Prominence HPLC system equipped with an RF-10AXL fluorescence detector (Shimadzu Corporation, Kyoto, Japan). Enzymatic oligonucleotide syntheses were performed using a WK-0518 Thermal cycler (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cells were cultivated in a 96-well tissue culture plate (Corning Japan K.K., Tokyo, Japan). Fluorescence microscopic images were captured using an inverted fluorescence microscope BZ-X710 (Keyence Corporation, Osaka, Japan).

2. Materials

Disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), and Dulbecco's Modified Eagle's Medium (DMEM) low glucose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Penicillin-Streptomycin Solution, Trypsin- EDTA solution, and streptavidin were purchased from Sigma-Aldrich, Inc. (MO, USA). Human thrombin and human fibrinogen were purchased from Merck K.K. (Tokyo, Japan). Fetal bovine serum (CELLect™ FBS, Gold) was purchased from MP CellTracker™ Green CMFDA Dye was purchased from Life Technologies Japan, Ltd. (Tokyo, Japan). *KOD Dash* DNA polymerase was purchased from Toyobo Co. Ltd. (Osaka, Japan). 2′-Deoxyadenosine triphosphate (dATP), 2′-deoxyguanosine triphosphate (dGTP) and 2′-deoxycytidine triphosphate (dCTP) were purchased from Roche Diagnostics K.K. (Tokyo, Japan). Oligonucleotides were purchased from Japan Bio Services Co. Ltd. (Saitama, Japan). HeLa cells (JCRB9004) and HepG2 cells (JCRB1054) were purchased from National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan).

3. Enzymatic synthesis of the carrier aptamer (bApt)

The carrier aptamer (bApt) was synthesized using a primer (TBA_P1), a template (T1), three natural 2′-deoxyribonucleoside triphosphates (dATP, dGTP, and dCTP), and a modified 2′-deoxyuridine triphosphate ($dU^{ad}TP$) catalyzed by the *KOD Dash* DNA polymerase (Table S1). The produced strand, bApt, was purified by poly-acrylamide gel electrophoresis (Fig. S2).[1]

4. Fluorescence polarization (FP) assay

4.1 Preparation of sample solutions

Thrombin was dissolved in distilled water, and then, a 4000 nM thrombin solution in 1× phosphate-buffered saline (PBS) (11.8 mM HPO_4^{2-}, 140 mM Cl^-, 157 mM Na^+, 4.5 mM K^+; pH 7.4) was prepared. Similarly, mother liquors of fibrinogen (10000 nM) and CPT1 (2000 nM) were prepared in 1× PBS.

A solution containing bApt (4000 nM) was prepared in 1× PBS, and then, bApt was refolded by annealing (preheating at 94°C for 0.5 min followed by cooling to 25°C at a rate of 0.5°C/min). Then, the bApt solution (100 µL, 4000 nM) was mixed with the thrombin solution (100 µL, 4000 nM). Subsequently, the CPT1 solution (200 µL, 2000 nM) was added and incubated at 25°C for 30 min to produce a ternary complex of thrombin, CPT1, and bApt (400 µL, 1000 nM). Thrombin-free solutions for control experiments were prepared by adding 1× PBS instead of the thrombin solution to the bApt solution. Similarly, bApt-free solutions for control experiments were prepared by adding 1× PBS instead of the bApt solution to the thrombin solution.

4.2 FP measurement for CPT1 entrapping by SOEF

Fibrinogen solutions were prepared in 1× PBS at six different concentrations (278, 556, 1111, 1667, 3333, and 6667 nM). To the fibrinogen solutions (90 µL), the ternary complex (thrombin/CPT1/bApt) solution (10 µL, 1000 nM) was added to give reaction mixtures (100 µL) containing the ternary complex (100 nM) at six different fibrin concentrations (250, 500, 1000, 1500, 3000, and 6000 nM). Next, the mixtures were incubated at 25°C for 30 min. FP measurements were performed using the LS-55 instrument, and data were recorded every 25 s for 5 min at 25°C with excitation at 490 nm and monitoring at 520 nm (Fig. 3).[2] Reaction mixtures without thrombin or bApt were prepared using the thrombin-free solution or the bApt-free solution instead of the ternary complex solution and were then incubated and analyzed (Fig. 3). Three independent experiments were performed.

5. Estimation of the rate of CPT1 incorporation into the fibrin gels

5.1 Preparation of sample solutions

Solutions of fibrinogen (10000 nM), thrombin (4000 nM), CPT1 (2000 nM), and bApt (4000 nM) were prepared in 1× PBS.

The bApt in solution (20 µL, 4000 nM) was refolded by annealing (preheating at 94°C for 0.5 min followed by cooling to 25°C at a rate of 0.5°C/min). The thrombin solution (20 µL, 4000 nM) and CPT1 solution (40 µL, 2000 nM) were successively added to the bApt solution, which was then

incubated at 25°C for 30 min to give a ternary complex (thrombin/CPT1/bApt) solution (80 μL, 1000 nM).

5.2 Determination of the amount of CPT1 in the supernatant

To a single well in a 96-well tissue culture plate, 160 μL of 1× PBS and 20 μL of the fibrinogen solution (10000 nM) were added and mixed by gentle pipetting. Subsequently, 20 μL of the ternary complex (thrombin/CPT1/bApt) solution (1000 nM) was added and mixed by gentle pipetting. Then, the solution (200 μL) was incubated under 95% air and 5% CO_2 at 37°C for 48 h to produce fibrin gels and supernatant. Thereafter, the supernatant (100 μL) was injected into an HPLC system (C_{18} reversed-phase column) equipped with a fluorescence detector with excitation at 490 nm and monitoring at 520 nm, and the amount of CPT1 in the supernatant was determined using a calibration curve, as described below (Fig. S8). Three independent experiments were performed. Under SOEF in the presence of 1000 nM fibrinogen and 100 nM thrombin, CPT1, and bApt, the CPT1 concentration in the supernatant was found to be 9.61 ± 0.56 nM, indicating that approximately 90% of CPT1 was incorporated into the fibrin gel.

5.3 Calibration curve for HPLC analysis

To obtain a calibration curve for HPLC analysis, CPT1 solutions were prepared in 1× PBS at four different concentrations (250, 500, 750, or 1000 nM) and incubated at 25°C for 30 min. To a single well in a 96-well tissue culture plate, 180 μL of 1× PBS and 20 μL of the CPT1 solution (250, 500, 750, or 1000 nM) were added and mixed. Furthermore, to obtain a solution without CPT1, 200 μL of 1× PBS was similarly added and mixed. Then, the five solutions were incubated under 95% air and 5% CO_2 at 37°C for 48 h. Thereafter, the solutions (100 μL) were injected into an HPLC system (C_{18} reversed-phase column) equipped with a fluorescence detector with excitation at 490 nm and monitoring at 520 nm to provide a calibration curve by quantifying the amounts of CPT1 in each solution (Fig. S8). Three independent experiments were performed.

6. Validation of the inhibitory effects of bApt as a drug carrier on cancer cell proliferation

6.1 Preparation of sample solutions

Solutions of fibrinogen (10000 nM), thrombin (4000 nM), CPT1 (2000 nM), bApt (4000 nM), and TBA (4000 nM) were prepared in 1× PBS.

The bApt and TBA in solution (100 μL, 4000 nM) were separately refolded by annealing (preheating at 94°C for 0.5 min followed by cooling to 25°C at a rate of 0.5°C/min). Then, to the bApt or TBA solution (100 μL, 4000 nM), the thrombin solution (100 μL, 4000 nM) and the CPT1 solution (200

μL, 2000 nM) were successively added, mixed, and incubated at 25°C for 30 min to give a solution of the ternary thrombin/CPT1/bApt complex (400 μL, 1000 nM) or a solution of the binary thrombin/TBA complex and free CPT1 (400 μL, 1000 nM each). Furthermore, for control experiments, solutions without one, two, or three of the three components, i.e., thrombin, CPT1, and aptamer (bApt or TBA), were prepared by using 1× PBS instead of the missing components.

6.2 Cell cultivation and cell growth inhibition assay

Trypsinized HeLa cells or HepG2 cells were diluted to a concentration of 1.0×10^4 cells/mL with a fresh medium containing DMEM-low glucose, a heat-inactivated serum (CELLect™ FBS, Gold), and an antibiotic solution (penicillin–streptomycin) at a percentage proportion of 90/9/1 (v/v/v %). The medium (300 μL) containing the cells was transferred to a 96-well tissue culture plate. Then, the cells were cultured in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 24 h. After 240 μL of the medium was replaced with a new medium, 30 μL of the fibrinogen solution (10000 nM) was added and mixed by gentle pipetting. Subsequently, the solution of the ternary thrombin/CPT1/bApt complex (30 μL, 1000 nM) was added and mixed by gentle pipetting. For control experiments, a solution of the binary thrombin/TBA complex and free CPT1 (30 μL, 1000 nM each) was utilized instead of the ternary complex solution. Similarly, the abovementioned solutions for the other control experiments were examined. Furthermore, control experiments without fibrinogen (replaced by 1× PBS) were conducted. The cells were cultured in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 48 h.

Next, 300 μL of the medium was replaced with 100 μL of medium containing DMEM-low glucose and CellTracker™ Green CMFDA dye in dimethyl sulfoxide (10 mM) at a percentage proportion of 99.8/0.2 (v/v %). The sample was then incubated in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 30 min. After 300 μL of the medium was replaced with 300 μL of 1× PBS, the stained cell layer was examined under a BZ-X710 inverted fluorescence microscope to acquire fluorescence images (40×) using excitation light (480 nm) and a cut-off filter (<520 nm) (Fig. 4 and S3–S6). Three independent experiments were performed.

7. Determination of the half-maximal inhibitory concentration (IC_{50})

7.1 Preparation of sample solutions

Solutions of fibrinogen (10000 nM), thrombin (4000 nM), CPT1 (2000 nM), and bApt (4000 nM) were prepared in 1× PBS. The bApt in solution (100 μL, 4000 nM) was refolded by annealing (preheating at 94°C for 0.5 min followed by cooling to 25°C at a rate of 0.5°C/min). Then, to the

solution of bApt (100 µL, 4000 nM), the thrombin solution (100 µL, 4000 nM) and CPT1 solution (200 µL, 2, 10, 20, 100, 150, 200, 500, 1000, or 2000 nM) were successively added and mixed, and the sample was incubated at 25°C for 30 min to give a solution (400 µL each) containing the three components, i.e., thrombin, CPT1, and bApt (1000 nM for thrombin and bApt and 1–1000 nM for CPT1). For control experiments without bApt, a solution (400 µL each) was prepared with 1× PBS instead of bApt (1000 nM for thrombin and 100–200000 nM for CPT1).

7.2 Cell cultivation and IC_{50} determination

Trypsinized HeLa cells were diluted to a concentration of 1.0×10^4 cells/mL with a fresh medium containing DMEM-low glucose, a heat-inactivated serum (CELLect™ FBS, Gold), and an antibiotic solution (penicillin–streptomycin) at a percentage proportion of 90/9/1 (v/v/v %). The medium (300 µL) containing the cells was transferred to a 96-well tissue culture plate. Then, the cells were cultured in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 24 h. After 240 µL of the medium was replaced with a new medium, 30 µL of the fibrinogen solution (10000 nM) was added and mixed by gentle pipetting. Subsequently, the abovementioned solution of the three components, i.e., thrombin, CPT1, and bApt (30 µL each, 1000 nM for thrombin and bApt and 1–1000 nM for CPT1), was added and mixed by gentle pipetting. For control experiments without bApt, a solution containing thrombin and CPT1 (30 µL each, 1000 nM for thrombin and 100–200000 nM for CPT1) was used instead of the solution with the three components. The cells were cultured in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 48 h.

Next, 300 µL of the medium was replaced with 100 µL of medium containing DMEM-low glucose and CellTracker™ Green CMFDA dye in dimethyl sulfoxide (10 mM) at a percentage proportion of 99.8/0.2 (v/v %). The sample was then incubated in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 30 min. After 300 µL of the medium was replaced with 300 µL of 1× PBS, the stained cell layer was examined under a BZ-X710 inverted fluorescence microscope to acquire fluorescence images (10×) using excitation light (480 nm) and a cut-off filter (<520 nm) (Fig. S7). The fluorescence intensity per cell count was obtained by analyzing the captured images using the Hybrid Cell Count analysis application of the BZ-X710 apparatus.[3] Three independent experiments were performed (Fig. 5).

8. Measurement of the fibrin gel thickness

8.1 Preparation of sample solutions

Solutions of fibrinogen (10000 nM), thrombin (4000 nM), and FAM-TBA (4000 nM) were prepared in 1× PBS. The FAM-TBA in solution (100 μL, 4000 nM) was refolded by annealing (preheating at 94°C for 0.5 min followed by cooling to 25°C at a rate of 0.5°C/min). Then, to the FAM-TBA solution (100 μL, 4000 nM), the thrombin solution (100 μL, 4000 nM) and 1× PBS (200 μL) were added and mixed, and the sample was then incubated at 25°C for 30 min to give a solution of the binary thrombin/FAM-TBA complex (400 μL, 1000 nM).

8.2 Thickness measurement

To a 96-well tissue culture plate, 240 μL of 1× PBS and 30 μL of the fibrinogen solution (10000 nM) were successively added and mixed by gentle pipetting. Subsequently, 30 μL of the solution of the binary thrombin/FAM-TBA complex (1000 nM) was added and mixed by gentle pipetting. Then, the solution (300 μL) was incubated under 95% air and 5% CO_2 at 37°C for 48 h to give fibrin gels and supernatant. After the supernatant was replaced with 300 μL of 1× PBS, the fibrin gel was examined under a BZ-X710 inverted fluorescence microscope to acquire fluorescence images (60×) using excitation light (480 nm) and a cut-off filter (<520 nm). Using Z-stack and the image-stitching functions of BZ-X710, 401 fluorescence images were acquired at intervals of 0.5 μm over a thickness of 200 μm along the z axis, giving a thickness of 46.2 ± 4.7 μm (Fig. S9).[4] Five independent experiments were performed.

References

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Table S1. Synthetic oligonucleotides used in this study.

ODN	5′-Modification	Sequence[a]
T1	6-FAM	CACGGCGAAGAAGTTACTCTGATACTATGACCACCCTACGTGTCTGGCGTGTCA CCCCAACCTGCCCTACCACGGA
TBA_P1	Non	TCCGTGGTAGGGCAGGTTGGGGTGA
29TBA	Non	AGTCCGTGGTAGGGCAGGTTGGGGTGACT
FAM-TBA[b]	6-FAM	AGTCCGTGGTAGGGCAGGTTGGGGTGACT

[a]Sequences are aligned in the 5′ to 3′ direction. [b](6-FAM)-labeled 29TBA.

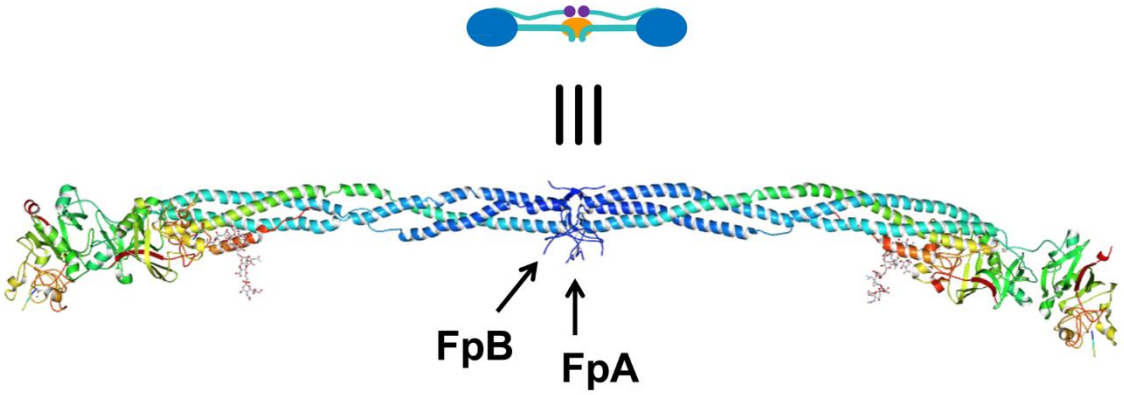


Fig. S1 Crystal structure of human fibrinogen (PDB: 3GHG)[5] and positions of the fibrinopeptides A and B (FpA and FpB).

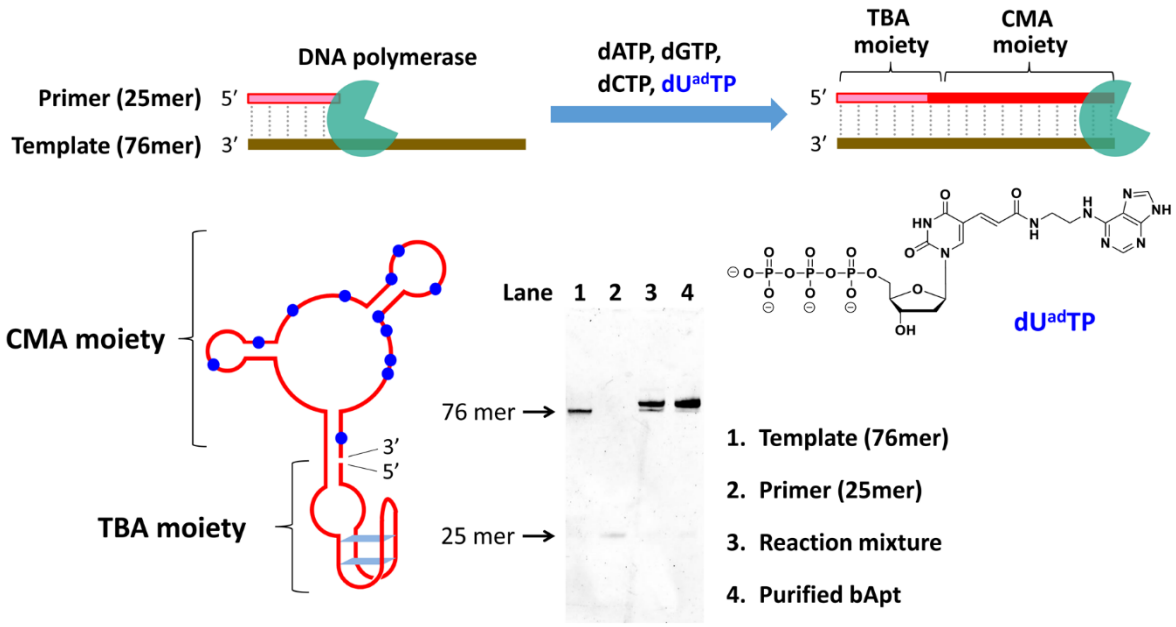


Fig. S2 Enzymatic synthesis of bApt by a primer extension reaction.

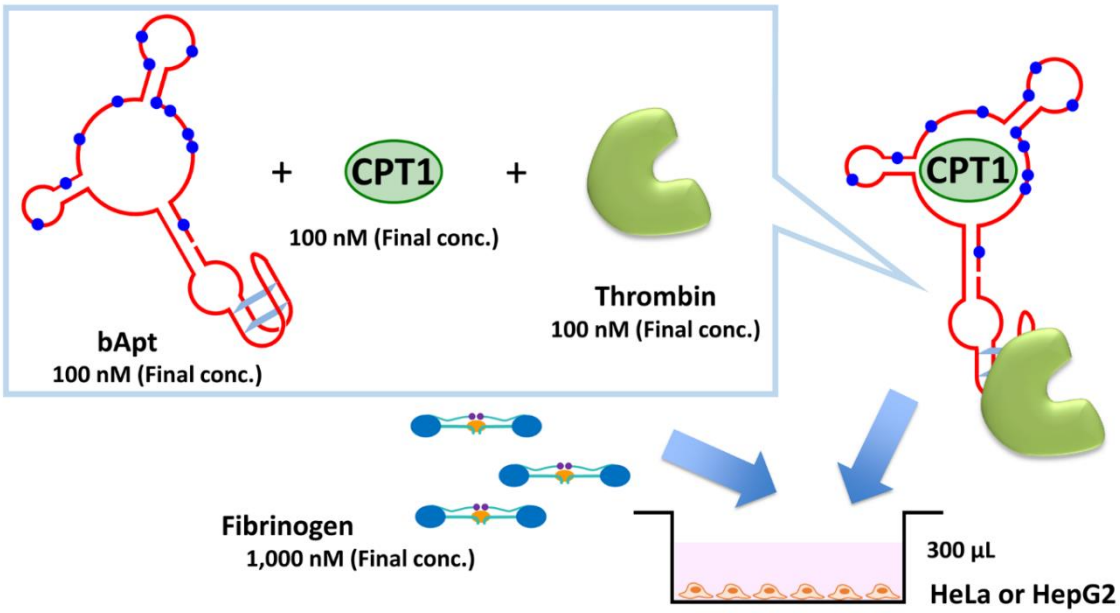


Fig. S3 Schematic of the cell growth inhibition assay. After the four components, i.e., fibrinogen, thrombin, CPT1, and bApt, were added, the cells were cultured in a humidified incubator containing 95% air and 5% CO_2 at 37°C for 48 h.

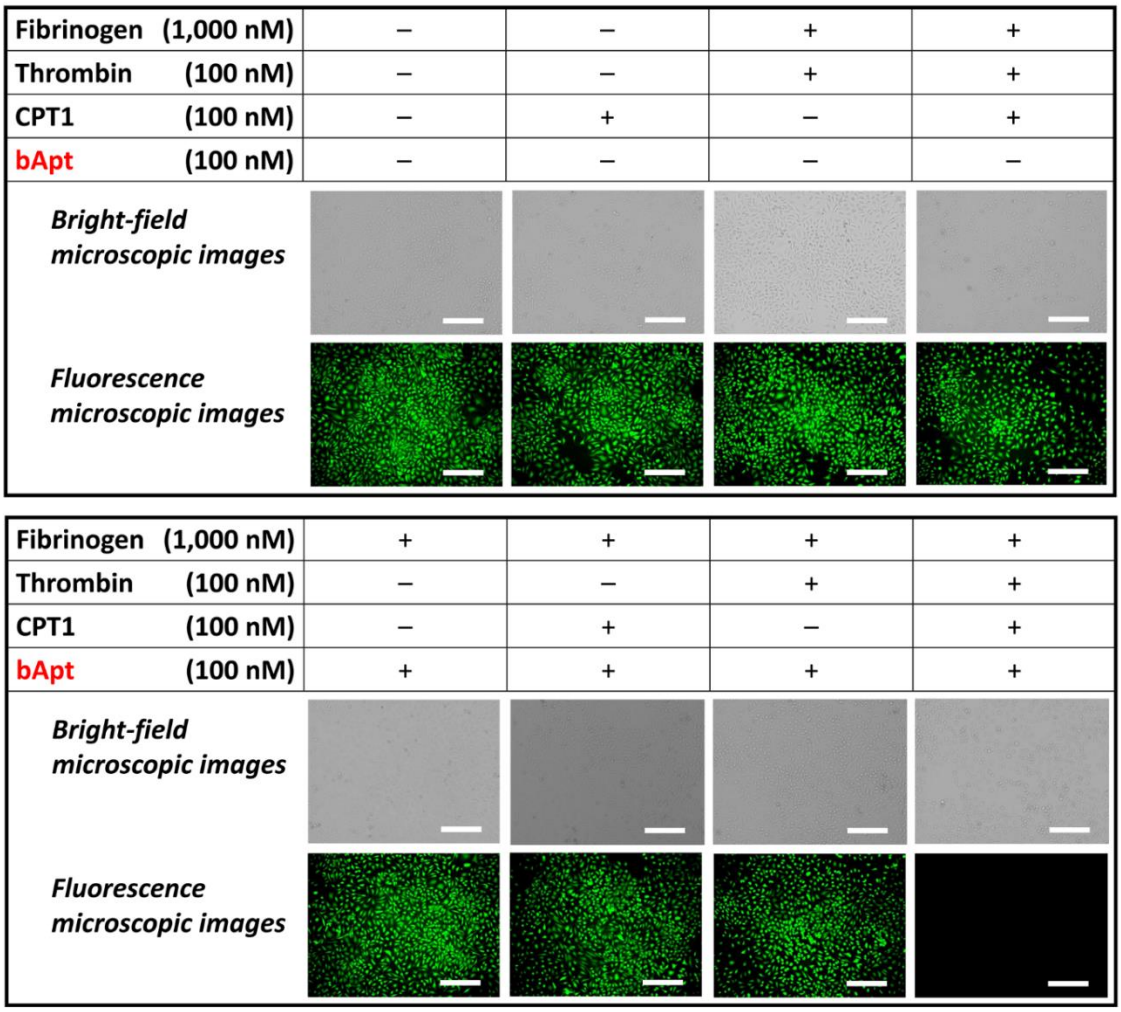


Fig. S4 Bright-field and fluorescence microscopy images of HeLa cells incubated for 48 h at 37°C after the addition of a solution containing the four components, i.e., fibrinogen, thrombin, CPT1, and bApt, or of a solution lacking one, two, three, or four of the four components. The fibrinogen concentration is 1000 nM, and the thrombin, CPT1, and bApt concentrations are each 100 nM. Scale bar = 50 μm.

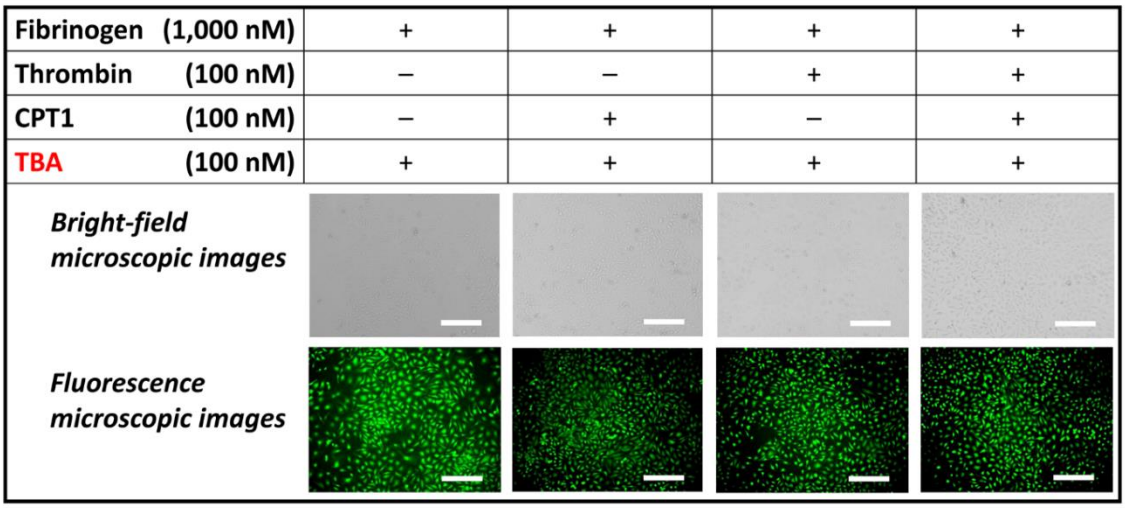


Fig. S5 Bright-field and fluorescence microscopy images of HeLa cells incubated for 48 h at 37°C after the addition of the fibrinogen solution and a solution containing three components, i.e., thrombin, CPT1, and TBA, or a solution lacking one, two, or three of the three components. The fibrinogen concentration is 1000 nM, and the thrombin, CPT1, and TBA concentrations are each 100 nM. Scale bar = 50 μm.

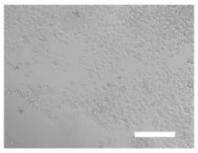
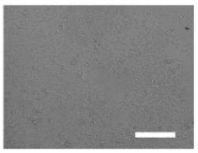
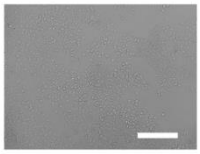
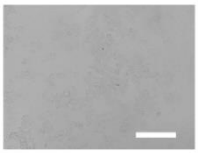
Fibrinogen (1,000 nM)	+	+	+	+
Thrombin (100 nM)	+	+	–	+
CPT1 (100 nM)	–	+	+	+
bApt (100 nM)	–	–	+	+
Bright-field microscopic images				

Fig. S6 Bright-field microscopy images of HeLa cells incubated for 48 h at 37°C after the addition of the fibrinogen solution and a solution containing three components, i.e., thrombin, CPT1, and bApt, or a solution lacking one of the three components. The fibrinogen concentration is 1000 nM, and the thrombin, CPT1, and bApt concentrations are each 100 nM. Scale bar = 50 μm.

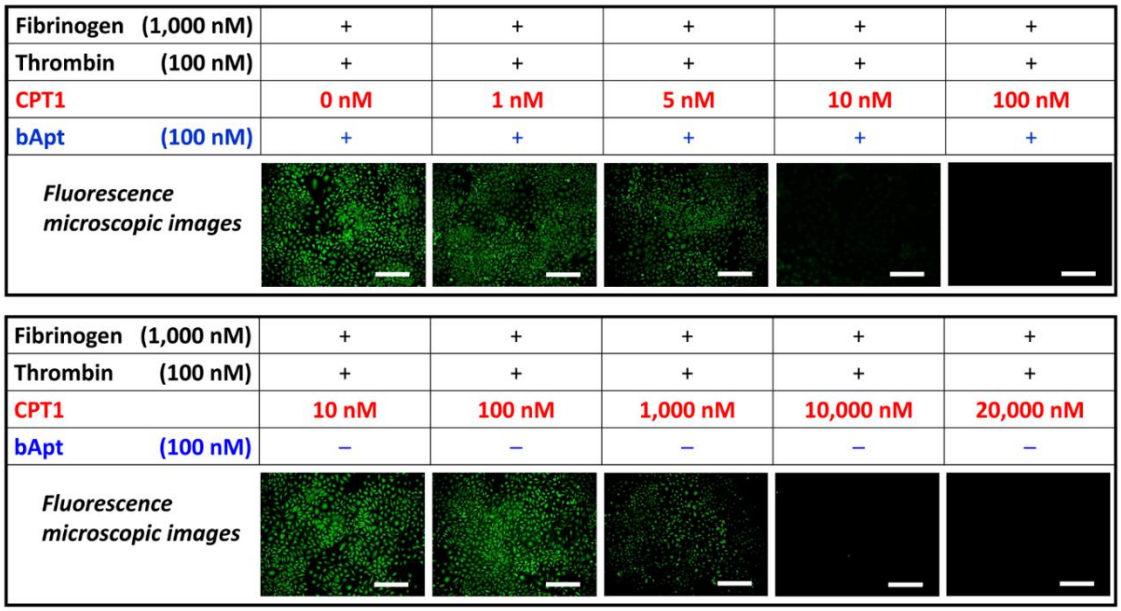


Fig. S7 Fluorescence microscopy images for IC_{50} determination with (top) or without (bottom) 100 nM of bApt at different CPT1 concentrations.

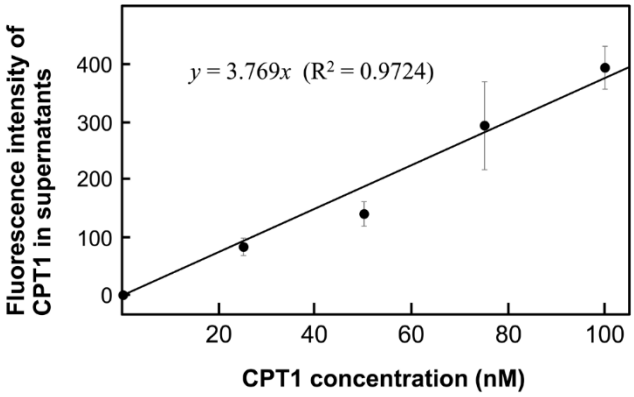


Fig. S8 Calibration curve provided by the amount of CPT1 in the supernatant after fibrin gel formation at different CPT1 concentrations.

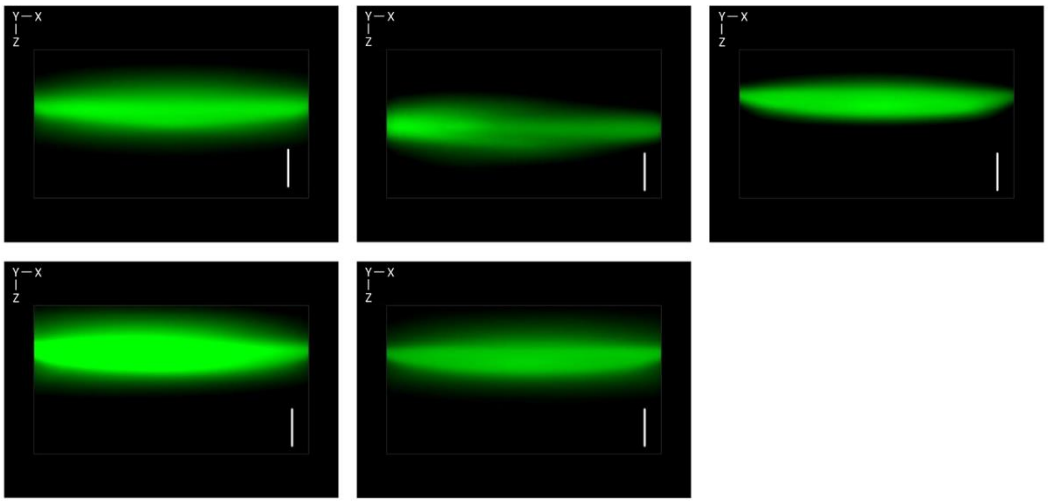


Fig. S9 Measurement of the thickness of fibrin gels containing the binary thrombin/FAM-TBA complex. Scale bar = 50 μm.