Supporting information

In-Situ Synthesis of an Anti-Cancer Peptide Amphiphile Using Tyrosine Kinase Overexpressed in Cancer Cells

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EXPERIMENTAL SECTION

Materials. Fmoc-amino acids, amino acid pre-loaded resins, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole-H₂O (HOBT-H₂O), triisopropylsilane (TIPS), and N,N-dimethylformamide (DMF) were purchased from Watanabe Chemical Industry (Hiroshima, Japan). Trifluoroacetic acid (TFA), lauric acid, myristic acid, dichloromethane (DCM), methanol, diethyl ether, acetonitrile, and 1-methyl-2-pyrrolidone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Piperidine, N,N-diisopropylethylamine (DIEA), decanoic acid, and 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Kaiser reagents for ninhydrin tests were purchased from Kokusan Chemical (Tokyo, Japan). Dulbecco’s phosphate-buffered saline (PBS), D-PBS (+) Preparation Reagent (Ca, Mg Solution) (100x), and 2.5 g/l-trypsin/1 mmol/l-EDTA solution were purchased from Nacalai Tesque (Kyoto, Japan). The phosphorylated peptide amphiphile was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

Solid-phase Synthesis of Peptide Amphiphiles. N-Acylated peptide amphiphiles were prepared by standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis on a 0.6 mmol scale. H-Glu(OtBu)-(Trt)-Trt(2-Cl)-resin was used as a polymeric support. A Fmoc-amino acid (4 equiv.) was coupled to the resin using HOBT-H₂O and DIEA as coupling agents in DMF for 60 min at room temperature. The resin was washed with DMF and DCM. The terminal Fmoc group was removed with DMF/piperidine (80/20), and the next Fmoc-amino acid was coupled. This process was repeated for each amino acid added to the growing peptide chain. The peptides synthesized on the resin were subjected to N-acylation with carboxylic acid (2.5 equiv.) in the presence of DIEA in DMF. Qualitative ninhydrin tests were used to confirm the completion of each coupling reaction. Deprotection and cleavage of the acylated peptides from the resin were performed in a mixture of TFA, TIPS, and water at a ratio of 95:2.5:2.5 for 90 min at room temperature. Acylated peptides in the cleavage mixture were precipitated with diethyl ether, collected by centrifugation, washed four times with diethyl ether, and freeze-dried under a vacuum.

N-Acylated peptides were purified by using a high-performance liquid chromatography (HPLC) system, LC-20AT (Shimadzu, Kyoto, Japan), equipped with a UV-vis detector SPD-20A and an Inertsil ODS-3 column (10 × 250 mm, GL Science, Tokyo, Japan). The eluent was controlled as a linear gradient of 0 to 100% acetonitrile in 0.1 M triethylamine-acetic acid (TEAA) buffer over 20 min. The eluted compounds were detected by absorbance at 280 nm. The retention time (min): 18.8 for C16-E4Y; 7.1 for C16E4; 5.2 for C16-E4F; 7.1 for C16-E4S; 9.5 for C8-E4Y; 12.8 for C12-E4Y; 10.0 for NBD-C8-E4Y; 10.2 for NBD-C8-E4pY. Purified products were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using an UltraflExtreme TM mass spectrometer (Bruker, Billerica, MA). MALDI-TOF/MS (DHB matrix, positive mode) (m/z) [M+Na]+, calcd. for C₁₆-E₄Y (C₄₃H₇₀N₅O₁₆), 958.46; found, 958.5; calcd. for C₁₆-EΕΕΕ (C₃₆H₆₀N₄O₁₄), 795.40; found, 795.4; calcd. for C₁₆-EΕΕΕ (C₃₆H₆₀N₄O₁₄), 795.40; found, 795.4; calcd. for C₁₆-E₄F (C₄₅H₆₀N₃O₁₃), 942.47; found, 942.5; calcd. for C₁₆-E₄S (C₃₆H₆₀N₄O₁₄), 882.43; found, 882.5; calcd. for C₈-E₄Y (C₃₇H₃₅N₅O₁₆), 846.34; found, 846.4; calcd. for C₁₂-E₄Y (C₄₁H₆₁N₅O₁₆), 902.40; found, 902.4.

Solid-phase Synthesis of NBD-C₈-E₄Y and NBD-C₈-E₄pY. Condensation of amino acids and Fmoc-Adod(8)-
OH was carried out using resin by the same protocol described above. To conjugate NBD-Cl with an N terminus of NH$_2$-C$_8$-peptide-resin, 1.8 mmol NBD-Cl, 680 µL DIEA, and 7.72 mL DMF were added to the resin and reacted for 24 h in darkness. The cleavage of the synthesized peptides from the resin was the same as described above. The peptide was purified using the HPLC system. The obtained product was identified by MALDI-TOF/MS (CHCA matrix, negative mode) ($m/z$). [M-H]$^-$ calc'd for NBD-C8-E4Y (C$_{43}$H$_{55}$N$_9$O$_{19}$), 1000.35; found, 1000.3; calc'd for NBD-C8-E4pY (C$_{43}$H$_{55}$N$_9$O$_{22}$P), 1080.32; found, 1080.1.

**Gelation Test.** PBS (+) was prepared supplementing D-PBS (+) Preparation Reagent (Ca, Mg Solution) (100x) into PBS. Peptide amphiphiles were dissolved in PBS (+) (containing Ca$^{2+}$ (0.90 mM) and Mg$^{2+}$ (0.49 mM)) at 1.0 wt% with heating at 60 °C in glass vials and then slowly cooled at room temperature for 15 min. Gel formation was evaluated by tilting the glass vials.

**Transmission Electron Microscope (TEM) Observations.** An elastic carbon-supported TEM grid was immersed in a peptide amphiphile solution of 1.0 wt% in PBS(+), dried, and stained with a 2.0 wt% phosphotungstic acid solution. TEM observations were performed using a JEM-2100F (JEOL, Tokyo, Japan) at an acceleration voltage of 200 kV.

**Cell Culturing.** Primary normal human dermal microvascular endothelial cells (MvE cells) were purchased from Cell Systems Corporation (Kirkland, WA). MvE cells were cultured in Complete Medium Kit with Serum and CultureBoost-R (Cell Systems Corporation). Four kinds of cell lines were used as cancer cell models. HeLa (human cervical carcinoma), MCF-7 (human breast adenocarcinoma), A431 (human epidermoid carcinoma), HepG2 (Human hepatocyte carcinoma), and HEK293 (human embryonic kidney) cells were kindly provided by Prof. A. Kondo (Kobe Univ.). MvE cells and HEK293 cells were used as normal cell models. These cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO) and a penicillin-streptomycin mixed solution (Nacalai Tesque). All cells were cultured at 37 °C in a humidified incubator with 5% CO$_2$ atmosphere.

**Phosphorylation Test of NBD-C8-E4Y.** NBD-C8-E4Y (100 µM), ATP (200 µM), and c-Src kinase (1 µg/mL) were dissolved in 1× kinase buffer (Cell Signaling Technology, Danvers, MA) containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na$_3$VO$_4$, and 10 mM MgCl$_2$. The solution was incubated at 37 °C, and the phosphorylation of NBD-C8-E4Y was periodically analyzed using HPLC. HPLC settings were as follows: eluent A, 0.1 M TEAA buffer; eluent B, acetonitrile; gradient, 0%–100% eluent B over 20 min; detection wavelength, 480 nm.

**Gel Formation of C16-E4Y Phosphorylated by Tyrosine Kinase.** C16-E4Y (1 wt%), ATP (15 mM), and c-Src kinase (6 µg/mL) were dissolved in 1× kinase buffer. The solution was incubated at 37 °C for 7 days.
**Tyrosine Kinase Activity Assay.** Cells were seeded on a 100-mm dish at a density of $1 \times 10^6$ cells/well and incubated overnight. The tyrosine kinase activity of the cells was determined using the Universal Tyrosine Kinase Assay Kit (Takara Bio Inc., Kusatsu, Japan). A microplate reader (SH9000, Hitachi High-Technologies Corporation, Tokyo, Japan) was used to measure the absorbance at 625 nm.

**Cytotoxicity Assay.** Cells were seeded into a 96-well plate (in five replicates) at a density of $1 \times 10^4$ cells/well. After incubation for 24 h, the medium was exchanged with a medium containing a peptide amphiphile (typically 0.05 wt%), and the cells were incubated for 20 h. WST-8 reagent (10 µL, Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) was added to each well, followed by incubation for 1 h at 37 °C. The absorbance at 450 nm was measured using the microplate reader.

**Tyrosine Kinase Inhibitor Assay.** Afatinib or erlotinib (Cayman Chemical Company, Ann Arbor, MI) were added at 200 nM to a medium culturing A431 cells on a microplate. After 24 h, the cytotoxicity assay of C16-E4Y (0.025 wt%) was performed without removing the tyrosine kinase inhibitors. Each cytotoxicity value was normalized by data without any additive (C16-E4Y and an inhibitor).

**Detection of a Peptide Amphiphile Phosphorylated in Cells.** A431 cells were incubated in a 60-mm dish at a density of $3 \times 10^5$ cells/dish for 24 h. After treatment with C16-E4Y (0.05 wt%) for another 24 h, the medium and the cell lysate were collected and analyzed by MALDI-TOF/MS.

**Fluorescence Enhancement of NBD-C8-E4Y via Co-Assembly with C16-E4Y.** C16-E4pY and NBD-C8-E4pY at 1.0 wt% and $5 \times 10^{-4}$ wt%, respectively, were dissolved in PBS (+) by heating. The solution became a hydrogel 24 h after at room temperature. The gel was mounted on a glass slide and covered by a coverslip. Green fluorescence of the specimen was observed using CLSM. Another sample prepared with C16-E4Y, instead of C16-E4pY, and NBD-C8-E4pY was used as a control.

**Cell Uptake of Peptide Amphiphiles.** A431 cells were seeded on a 35-mm glass-base dish at a density of $1.0 \times 10^5$ cells/dish and incubated for 24 h. The cells were treated with a medium containing 0.05 wt% C16-E4Y (or C16-E4pY) and 0.001 wt% NBD-C8-E4Y. After 3 h, the cells were stained with Hoechst 33342 for 15 min, followed by two wash steps, and the medium was exchanged with Live Cell Imaging Solution (Thermo Fisher Scientific, Waltham, MA). The green fluorescence of NBD was observed using a confocal laser scanning microscope (CLSM) (FV3000, Olympus, Tokyo, Japan).

**Localization of Peptide Amphiphiles in Cells.** A431 cells were seeded on a 35-mm glass-base dish at a density of $1.0 \times 10^5$ cells/dish. After incubation for 24 h, the cells were treated with a medium containing C16-E4Y (0.05 wt%) and NBD-C8-E4Y (0.001 wt%). After incubation for a further 24 h, the cells were stained with 2 µL ER tracker™ Red (Thermo Fisher Scientific, Waltham, MA) for 15 min. Mitochondria were stained using Cellstain® MitoRed
(Dojindo Laboratories) for 1 h. The medium was exchanged with Live Cell Imaging Solution, and cells were observed using the CLSM.

**Fluorescence Recovery After Photobleaching (FRAP) Assay.** A431 cells were seeded on a 35-mm glass-base dish at a density of $1.0 \times 10^5$ cells/dish. After incubation for 24 h, the cells were treated with a medium containing 0.08 wt% C16-E4Y. After further incubation for 1 h, the cells were stained with 2 µL ER tracker™ Green (Thermo Fisher Scientific) for 15 min. A FRAP assay was performed using a CLSM as follows. A circled area ($1.67 \mu m^2$) on ER was photobleached with a 365 nm laser for 200 µs. Green fluorescence images were captured just before and after the photobleaching.

**Apoptosis and Necrosis Detection.** $2.0 \times 10^5$ cells were incubated for 24 h on a 35-mm glass-base dish. The medium was exchanged with a medium containing C16-E4Y (0.08 wt%), and the cells were incubated for 1 h. FITC-annexin V, ethidium homodimer III, and Hoechst 33342 (Apoptotic/Necrotic/Healthy Cells Detection Kit; PromoCell, Heidelberg, Germany) were added to the cell medium, and then green/red/blue fluorescence images were observed using the CLSM after incubation for 15 min. Percentages of cells in early apoptosis and late apoptosis or necrosis were calculated by counting at least 1000 cells in the CLSM images.

**In Vivo Experiments.** All animal experiments were carried out at Japan SLC in accordance with the institutional guideline for the care and use of laboratory animals (approval no. F71-8133). Six-week-old female nude mice (BALB/cSlc-nu/nu) were purchased from Japan SLC (Shizuoka, Japan) and maintained under standard conditions for one week. A431-xenografted mice were prepared by subcutaneously injecting 100 µL of a suspension of A431 cells ($1 \times 10^7$ cells) into the flank. On day 7, after the inoculation, the mice were randomly assigned to two groups ($n=6$): (i) PBS and (ii) PBS containing 1.5 wt% C16-E4Y. The sample solutions (30 µL) were directly injected into the tumor three times a week for three weeks. Tumor sizes were measured three times a week using a caliper, and tumor volumes were calculated using the following formula:

\[ \text{Tumor volume (mm}^3\text{)} = \text{longer diameter} \times \text{shorter diameter}^2 \times 0.5 \]

Mice were sacrificed under anesthesia with isoflurane four weeks after inoculation.
RESULTS
MALDI-TOF/MS Analysis of Peptide Amphiphiles Synthesized

![Graphs showing MALDI-TOF/MS analysis results for C16-E4Y, C16-E4, C16-E4F, and C16-E4S.]
Figure S1. MALDI-TOF/MS spectra of (a) C16-E4Y, (b) C16-E4, (c) C16-E4F, (d) C16-E4S, (e) C8-E4Y, (f) C12-E4Y, (g) NBD-C8-E4Y, and (h) NBD-C8-E4pY. (a–f) 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix in the positive mode. m/z of [M+H]⁺ calcd. for C₁₆-E₄Y (C₴₅H₇₀N₅O₁₆), 958.46; found, 958.5; calcd. m/z of [M+H]⁺ calcd. for C₁₆-E₄ (C₄₅H₇₀N₅O₁₆), 795.40; found, 795.4. m/z of [M+H]⁺ calcd. for C₁₆-E₄F (C₴₅H₇₀N₅O₁₅), 942.47; found, 942.5. m/z of [M+H]⁺ calcd. for C₁₆-
E4S (C_{39}H_{65}N_{18}O_{16}), 882.43; found, 882.5. [M+H]^+ calcd. for C_{37}H_{53}N_{18}O_{16}, 846.34; found, 846.4. [M+H]^+ calcd. for C_{12}-E4Y (C_{47}H_{60}N_{18}O_{16}), 902.40; found, 902.4. (g, h) α-Cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix in the negative mode. m/z of [M-H]^- calcd for NBD-C8-E4Y (C_{43}H_{55}N_{9}O_{19}), 1000.35; found, 1000.3. m/z of [M-H]^- calcd for NBD-C8-E4pY (C_{43}H_{55}N_{9}O_{22}P), 1080.32; found, 1080.1.

**HPLC Analysis of Peptide Amphiphiles Synthesized**
**Figure S2.** HPLC chromatogram of (a) C16-E4Y, (b) C16-E4, (c) C16-E4F, (d) C16-E4S, (e) C8-E4Y, (f) C12-E4Y, (g) NBD-C8-E4Y, and (h) NBD-C8-E4pY. The retention time (min): 18.8 for C16-E4Y; 7.1 for C16-E4; 5.2 for C16-E4F; 7.1 for C16-E4S; 9.5 for C8-E4Y; 12.8 for C12-E4Y; 10.0 for NBD-C8-E4Y; 10.2 for NBD-C8-E4pY.

**Phosphorylation of NBD-C8-E4Y by Tyrosine Kinase**

**Figure S3.** HPLC profiles for the phosphorylation of NBD-C8-E4Y catalyzed by tyrosine kinase (c-Src). NBD-C8-E4Y (at a final concentration of 100 µM) was dissolved in 1× kinase buffer supplemented with 200 µM ATP, followed by the addition of c-Src kinase (at a final concentration of 1 µg/mL) to start the phosphorylation reaction.
Gel Formation of C16-E4Y Phosphorylated by Tyrosine Kinase

Figure S4. C16-E4Y solution incubated (a) with and (b) without a tyrosine kinase and ATP for 7 days.

Detection of a Peptide Amphiphile Phosphorylated in A431 Cells

Figure S5. MALDI-TOF/MS analysis of a cell-culture medium and cell lysate. (a) Medium (DMEM) in which A431 cells were cultured with 0.05 wt% C16-E4Y. (b) A431 cell lysate after culturing with 0.05 wt% C16-E4Y. The solid arrow and dashed arrows indicate C16-E4Y and C16-E4pY, respectively. (c) DMEM in which A431 cells were cultured in the absence of C16-E4Y. (d) A431 cell lysate after culturing in the absence of C16-E4Y. Calcd. m/z for [C16-E4YNa]^+, 958.5; found 959.4. Calcd. m/z for [C16-E4pYNa]^+, 1038.4; found 1038.7.
Detection of a Peptide Amphiphile in HepG2 Cells

Figure S6. MALDI-TOF/MS analysis of a cell-culture medium and cell lysate of HepG2 cells. (a) Medium (DMEM) in which HepG2 cells were cultured with 0.05 wt% C16-E4Y. (b) HepG2 cell lysate after culturing with 0.05 wt% C16-E4Y. The solid arrow indicates C16-E4Y. (c) DMEM in which HepG2 cells were cultured in the absence of C16-E4Y. (d) HepG2 cell lysate after culturing in the absence of C16-E4Y. Calcd. m/z for [C16-E4YNa]+, 958.5; found 958.4.

Cytotoxicity of C16-E4pY

Figure S7. Cytotoxicity of C16-E4pY to five different cell lines. Cells were cultured with C16-E4pY for 24 h,
followed by cell viability assays using Cell Counting Kit-8. C16-E4pY did not show remarkable cytotoxicity toward any cell line tested, whereas C16-E4Y showed dose-dependent cytotoxicity toward A431 cells (Figure 3b).
Cytotoxicity of C16-E4Y Analogs

**Figure S8.** Cytotoxicity of peptide amphiphile analogs toward five different cell lines. (a) C16-E4, (b) C16-E4F, and (c) C16-E4S. Cells were cultured with the peptide amphiphile for 24 h, followed by cell viability assays.

**Figure S9.** Cytotoxicity of (a) C8-E4Y and (b) C12-E4Y toward four different cell lines. Cells were cultured with the peptide amphiphile for 24 h, followed by cell viability assays.
TEM Observation of Peptide Amphiphiles with a Short Acyl Chain

Figure S10. TEM images of (a) C8-E4Y solution, (b) C12-E4Y solution, (c) C8-E4pY solution, and (d) C12-E4pY solution.

Fluorescence Enhancement of NBD-C8-E4Y via Co-Assembly with C16-E4Y

Figure S11. CLSM images of (a) a hydrogel formed by 1 wt% C16-E4pY and $5 \times 10^{-4}$ wt% NBD-C8-E4pY and (b) a mixture solution of 1 wt% C16-E4Y and $5 \times 10^{-4}$ wt% NBD-C8-E4pY.
Localization of a Peptide Amphiphile in Cells

**Figure S12.** CLSM images of A431 cells stained with MitoRed after incubation with a mixture of NBD-C8-E4Y (0.001 wt%) and C16-E4Y (0.05 wt%) for 24 h. Green, NBD-C8-E4Y; red, MitoRed. Scale bar, 100 µm.

Cell Uptake of a Peptide Amphiphile

**Figure S13.** CLSM images of A431 cells incubated with/without peptide amphiphiles 3 h after incubation. (a–c) Green fluorescence images (NBD). (d–f) Bright-field images. (g–i) Merged images of bright field, green fluorescence, and blue fluorescence (Hoechst 33342) images. (a, d, g) Without a peptide amphiphile. (b, e, h) With NBD-C8-E4pY and C16-E4pY. (c, f, i) With NBD-C8-E4Y and C16-E4Y. Scale bars represent 15 µm. The absence of green fluorescence in Figure S8b and h indicates that cells did not take up C16-E4pY, a phosphorylated peptide amphiphile.
Fluorescence Recovery After Photobleaching (FRAP) Assay

Figure S14. Fluorescence recovery after photobleaching (FRAP). A431 cells were not treated (a, b) or treated (c, d) with 0.08% C16-E4Y for 1 h. The cells were stained with ER-tracker green (BODIPY™ FL glibenclamide) just before FRAP observation. Fluorescence of ER-tracker was observed (a, c) before and (b, d) 1 s after photobleaching using CLSM. Red circles indicate areas that underwent photobleaching.
Apoptosis and Necrosis Detection

**Figure S15.** Apoptosis and necrosis assay for the cells after incubation (a) with and (b) without C16-E4Y for 1 h. (i) A431 cells and (ii) HeLa cells. Typical CLSM images (low magnification). Scale bar, 150 µm; green, annexin V-FITC; red, propidium iodide; blue, Hoechst 33342. (c) Percentages of cells in early apoptosis and in late apoptosis or necrosis. Data were calculated by counting at least 1000 cells in CLSM images.