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Article

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

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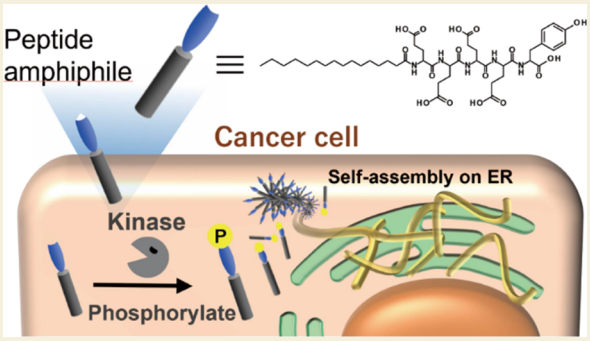
ABSTRACT: Cell-selective killing using molecular self-assemblies is an emerging concept for cancer therapy. Reported molecular self-assemblies are triggered by hydrolysis of well-designed molecules inside or outside cancer cells. This hydrolysis can occur in cancer and normal cells because of the abundance of water in living systems. Here, we report the in situ synthesis of a self-assembling molecule using a tyrosine kinase overexpressed in cancer cells. We designed a tyrosine-containing peptide amphiphile (C16-E4Y) that is transformed into a phosphorylated peptide amphiphile (C16-E4pY) by the overexpressed tyrosine kinase. Phosphorylation of C16-E4Y promoted self-assembly to form nanofibers in cancer cells. C16-E4Y exhibited selective cytotoxicity toward cancer cells overexpressing the tyrosine kinase. Self-assembled C16-E4pY induced endoplasmic reticulum stress that caused apoptotic cell death. Animal experiments revealed that C16-E4Y has antitumor activity. These results show that an enzyme overexpressed in cancer cells is available for intracellular synthesis of an antitumor self-assembling drug that is cell-selective.



KEYWORDS: *low-molecular-weight gelator, self-assembly, tyrosine kinase, anticancer drug, peptide lipids*

INTRODUCTION

Molecular self-assembly has been studied widely as a carrier for drug delivery to avoid side effects and increase drug efficacy, especially in cancer therapy.[1−5] In the last decade, intracellular self-assembly of small synthetic molecules has emerged as a novel approach to kill cancer cells selectively.[6−11] In this approach, the self-assembly of synthetic molecules is mainly triggered by hydrolytic enzymes (e.g., phosphatase, esterase, and proteinase) that are overexpressed in some cancer cells. Because these hydrolytic enzymes are ubiquitous in a living system and there is an abundance of water, precursors can always undergo hydrolysis somewhere throughout the body, resulting in nonspecific cytotoxicity. Thus, in this study, we present a novel approach for synthesizing a self-assembling molecule inside cancer cells that induces the selective killing of cancer cells (Figure 1a).

Although hydrolytic reactions are dominant in an aqueous environment, various molecules are synthesized from monomers and building blocks in a living cell using adenosine triphosphate (ATP) and intracellular enzymes. Tyrosine kinases catalyze the phosphorylation of tyrosine residues in proteins. Some cancer cells overexpress receptor tyrosine kinases,[12−15] which play important roles in cell growth, suppression of apoptosis, and cell invasion. Inhibition of kinases in cancer cells is a rational approach to treating cancer. Indeed, a series of molecularly targeted drugs have been

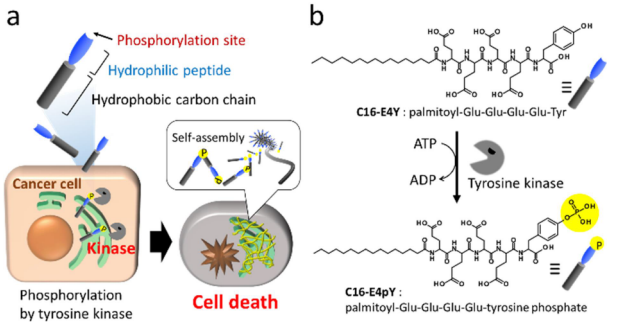


Figure 1. (a) Selective killing of cancer cells based on the self-assembly of a phosphorylated peptide amphiphile synthesized by a tyrosine kinase overexpressed in cancer cells. (b) Molecular structures of the peptide amphiphile (C16-E4Y) and its phosphorylated form (C16-E4pY).

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developed and approved for cancer therapy in several countries since the end of the last century.[16−18] Despite high expectations, long-term administration of these drugs often leads to drug resistance in cancer cells, which is typically caused by kinase mutations.[18−20]

The present study provides another approach to killing cancer cells selectively based on the overexpression of a tyrosine kinase, which is profoundly different from conventional molecularly targeted drugs. We synthesized a self-assembling molecule using ATP and a tyrosine kinase overexpressed in cancer cells. Kinase-catalyzed synthesis only occurs inside living cells where ATP and kinases reside. We designed a tyrosine-containing peptide amphiphile (C16-E4Y, Figure 1b), which was inspired from a substrate for a tyrosine kinase.[21] The tyrosine kinase phosphorylates C16-E4Y in cancer cells to yield C16-E4pY, which self-assembles to form nanofibers in cancer cells that induce acute stress and ultimately cell death.

RESULTS AND DISCUSSION

Design and Synthesis of Peptide Amphiphiles

To realize the concept in Figure 1a, we designed a peptide amphiphile (C16-E4Y: *N*-palmitoyl-Glu-Glu-Glu-Glu-Tyr) that contained a palmitoyl group as a long hydrophobic chain and a tyrosine kinase substrate sequence as a hydrophilic moiety (Figure 1b). C16-E4Y and its analogs tested in this study were prepared using solid-phase synthesis and confirmed by matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) and high-performance liquid chromatography (HPLC) (Figures S1 and S2). Prior to experiments using cells, we confirmed that a tyrosine kinase (c-Src) catalyzed the phosphorylation of the peptide sequence using a fluorescence-labeled analog (NBD-C8-E4Y) in the presence of ATP (Figure S3).

Gelation Test

Gelation tests revealed that 1.0 wt % C16-E4Y did not induce gelation of phosphate-buffered saline supplemented with 0.95 mM Ca^{2+} and 0.49 mM Mg^{2+} (PBS(+)) (Figure 2a). Phosphorylated C16-E4Y (C16-E4pY) at 1.0 wt % resulted in gelation of PBS(+) (Figure 2c). In the absence of the divalent metal ions, 1.0 wt % C16-E4pY did not form a hydrogel, indicating that complex formation of divalent metal ions with phosphate groups of Tyr residues facilitated the gelation process. TEM observations revealed that C16-E4Y formed nanosized strings several hundred nanometers in length, which were separated from each other (Figure 2b). C16-E4pY formed entangled or branched nanofibers whose lengths exceeded several micrometers (Figure 2d). When C16-E4Y was incubated with a tyrosine kinase and ATP to enzymatically produce C16-E4pY, the solution was partially gelated (Figure S4). These results indicated that phosphorylation of C16-E4Y by a tyrosine kinase caused C16-E4pY to self-assemble and form entangled nanofibers.

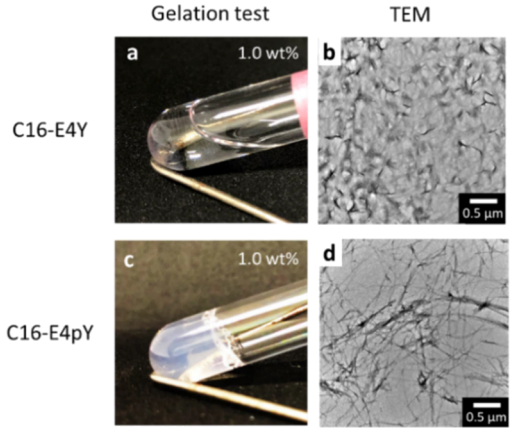

Figure 2. (a, c) Gelation tests of C16-E4Y and C16-E4pY solutions. Phosphate-buffered saline supplemented with 0.95 mM Ca^{2+}, and 0.49 mM Mg^{2+} was used. (b, d) Transmission electron microscope (TEM) observations of these solution.

Cytotoxicity Assay

We then assessed the tyrosine kinase activity of four different cancer cell lines (HeLa, A431, MCF-7, and HepG2 cells) and two different types of normal cells (MvE and HEK293 cells). A431 cells exhibited the highest tyrosine kinase activity among the several cell lines examined (Figure 3a). Numerous reports describe that A431 cells overexpress the epidermal growth factor receptor (EGFR), which functions as a tyrosine kinase.[22]

Cytotoxicity assays for C16-E4Y and C16-E4pY were conducted using these cell lines. C16-E4Y at 0.05 wt % showed remarkably higher cytotoxicity toward A431 cells when compared with that of the other cell lines. The cytotoxicity observed was dose-dependent (Figure 3b). MALDI-TOF/MS analysis found C16-E4pY in both the A431 cell lysate and cell-cultured medium, in which A431 cells were incubated with 0.05 wt % C16-E4Y (Figure S5). Phosphorylation of C16-E4Y was not detected in either the cell lysate or cultured medium of HepG2 cells that expressed a low level of a tyrosine kinase (Figure S6). These results showed the successful phosphorylation of C16-E4Y in A431 cells. Interestingly, C16-E4pY did not show remarkable cytotoxicity toward any cell lines at the same concentration, as shown in Figure 3b (Figure S7).

We then synthesized another three different kinds of peptide amphiphiles, which were unable to be phosphorylated by tyrosine kinase. C16-E4 (*N*-palmitoyl-Glu-Glu-Glu-Glu) did not have a tyrosine residue. C16-E4F (*N*-palmitoyl-Glu-Glu-Glu-Glu-Phe) did not have a phenolic hydroxy group on an aromatic ring. C16-E4S (*N*-palmitoyl-Glu-Glu-Glu-Glu-Ser) was designed to have a serine residue instead of a Tyr residue. These peptide amphiphiles did not exhibit selective cytotoxicity to A431 cells (Figure S8).

We also synthesized different analogs with short acyl chains and evaluated their cytotoxicity. Both C8-E4Y (*N*-capryloyl-Glu-Glu-Glu-Glu-Tyr) and C12-E4Y (*N*-lauroyl-Glu-Glu-Glu-Glu-Tyr) did not show cytotoxicity even at 0.1 wt % toward all cell lines tested (Figure S9). The self-assembling ability of a peptide amphiphile has been shown to decrease as the acyl chain length decreases.[23] Indeed, TEM observation confirmed their poor self-assembling ability. C8-E4Y did not form any self-assembly body (Figure S10a). C12-E4Y formed only a few isolated short fibers (Figure S10b). C8-E4pY formed a needle-like assembly (Figure S10c). C12-E4pY formed long but very few strands (Figure S10d). Thus, the weaker self-assembling ability of C8-E4Y and C12-E4Y may explain their non-cytotoxicity.[11,24]

The importance of intracellular phosphorylation by a tyrosine kinase was examined by adding two different EGFR inhibitors (afatinib and erlotinib)[19,25−27] to the cytotoxicity tests of C16-E4Y. Both EGFR inhibitors alleviated the cytotoxicity of 0.025 wt % C16-E4Y to A431 cells (Figure

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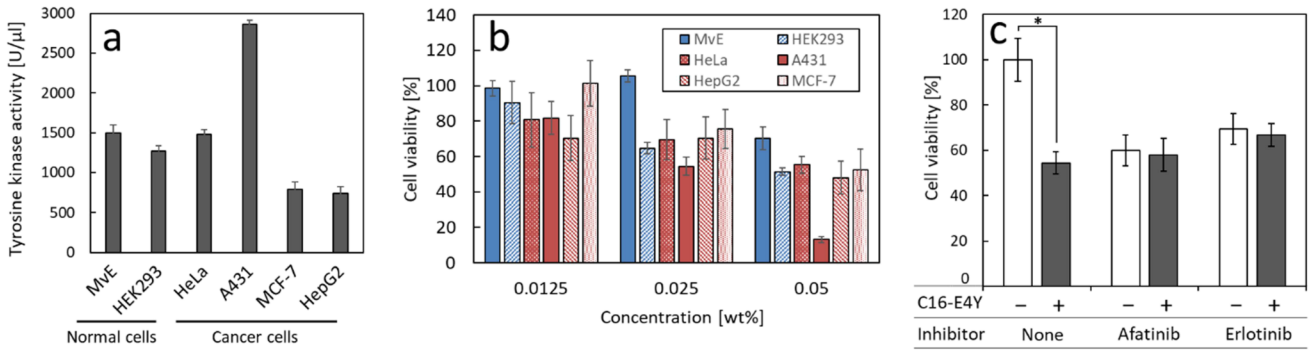

Figure 3. (a) Tyrosine kinase activity of the various cell lines tested. (b) Cytotoxicity of C16-E4Y toward the various cell lines tested. (c) Effect of EGFR inhibitors (afatinib and erlotinib, 200 nM) on the cytotoxicity of C16-E4Y 0.025 wt % to A431 cells (*$p < 0.01$). Cell viability without any additives (C16-E4Y and inhibitors) was taken as 100%.

3c). Although 0.025 wt % C16-E4Y resulted in 53% cell viability, the coexistence of C16-E4Y and inhibitors yielded comparable cell viability. Notably, cell viability decreased to 60−70% when only the inhibitor was added to the culture of A431 cells. These results indicate the catalytic function of EGFR in the cytotoxicity of C16-E4Y.

Localization in Cells

NBD-C8-E4Y was synthesized to identify the cellular uptake and location of C16-E4Y in cells. 7-Nitrobenzofurazan (NBD) is a fluorophore that emits green fluorescence in the self-assembly of hydrophobic molecules (Figure S11). The Xu group and our group have reported green fluorescence in the nanofibrous co-assembly of an NBD-labeled peptide amphiphile and a peptide amphiphile.[7,28] A431 cells were observed by confocal laser scanning microscopy (CLSM) after adding a mixture of NBD-C8-E4Y and C16-E4Y to the culture. Endoplasmic reticulum (ER) and mitochondria were stained with ER-tracker and Mito-tracker, respectively. Green fluorescence of the peptide amphiphile overlapped with red fluorescence of ER-tracker (Figure 4), whereas Mito-tracker

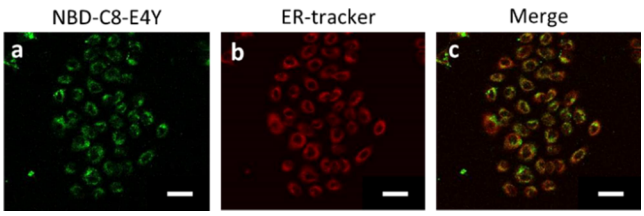

Figure 4. CLSM images of A431 cells after incubation with NBD-C8-E4Y (0.001 wt %, green) and C16-E4Y (0.05 wt %) for 24 h. The cells were stained with ER-tracker (red). Scale bar: 50 μm.

did not colocate with green fluorescence (Figure S12). Because there are reports describing that an acyl chain conveys an acylated molecule to a specific organelle in living cells,[29] C16-E4Y was also conveyed and accumulated on ER selectively. A similar accumulation of another peptide amphiphile on ER was observed in our previous study.[11]

Another fluorescence-labeled analog of C16-E4pY (NBD-C8-E4pY) was also synthesized, and its cell uptake was studied. We found that the uptake of NBD-C8-E4pY by A431 cells did not occur (Figure S13) probably because of electrostatic repulsion between the negatively charged cell surface and the negatively charged phosphate group of C16-E4pY. Variation in the cell uptake of the peptide amphiphiles may account for the difference in cytotoxicity between C16-E4Y and C16-E4pY (Figures 3b and S7).

The self-assembly of C16-E4Y accumulated on ER was studied using the fluorescence recovery after photobleaching (FRAP) assay. A small area of ER in A431 cells treated with C16-E4Y and ER-tracker was photobleached by laser. The fluorescence of ER-tracker without C16-E4Y recovered immediately within 1 s after photobleaching. In contrast, the fluorescence with C16-E4Y recovered relatively slowly (Figure S14). The delay in fluorescence recovery indicates a decrease in the fluidity of the ER membrane caused by the self-assembly of C16-E4Y accumulated on the ER membrane. ER is the largest store of releasable Ca^{2+} in cells, and the Ca^{2+} concentration is maintained at ∼1 mM in ER,[30,31] which might also contribute to the self-assembly of C16-E4Y on the ER.

Apoptosis and Necrosis Detection

Next, the mode of cell death caused by C16-E4Y was investigated using annexin V-FITC and propidium iodide (PI) to detect apoptosis or necrosis using CLSM (Figure S15). Green fluorescence derived from annexin V-FITC was observed on cell membranes after cell incubation with C16-E4Y for 1 h, indicating that the cells were in the early stages of apoptosis. Red fluorescence derived from PI indicated cellular membrane damage, and thus dying or dead cells. The coincidence of green and red fluorescence on the cells suggested that A431 cells died through apoptosis in the presence of C16-E4Y (Figure S15a(i)). CLSM observation of HeLa cells showed no staining with annexin V-FITC and PI (Figure S15a(ii)), indicating almost no apoptosis and no cell death. Only a few cells were dead among A431 and HeLa cells not treated with C16-E4Y (Figure S15b(i) and (ii)). A statistical analysis using CLSM images showed that 15.4% of A431 cells and 0.4% of HeLa cells were in early apoptosis (Figure S15c). Several studies reported that the accumulation of unfolded proteins on the ER causes ER stress and induces apoptosis.[32−35] In the present study, the accumulation of C16-E4Y on the ER would also trigger apoptosis. Our results suggest that C16-E4Y was taken up by A431 cells and phosphorylated by the tyrosine kinase overexpressed in cells. The produced C16-E4pY self-assembles to form nanofibers on the ER with divalent cations (Ca^{2+} and Mg^{2+}) in cells. The resulting ER stress induced apoptosis of the A431 cells.[36]

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Antitumor Activity In Vivo

Finally, the antitumor effect of C16-E4Y in vivo was examined. Nude mice subcutaneously xenografted with A431 cells were prepared. PBS (30 μL) containing C16-E4Y (1.5%) was injected locally in the tumor three times a week for three weeks. For treated mice, tumor growth was suppressed significantly, and the bodyweights of these mice were comparable to those of the control group (Figure 5).

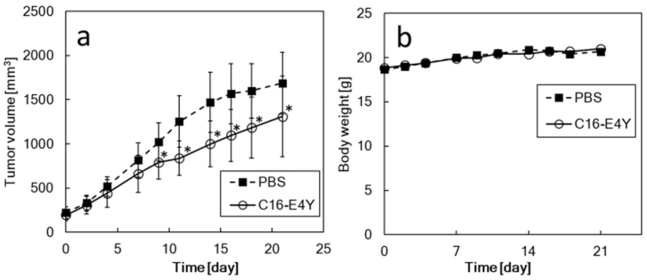

Figure 5. (a) Effect of C16-E4Y on A431 tumor growth by local injection into the tumor in mice (*$p < 0.05$). (b) Bodyweight change of mice during the administration experiments. $n = 6$.

CONCLUSIONS

We demonstrated that tyrosine kinase overexpressed in cancer cells transformed the designed peptide amphiphile, C16-E4Y, to synthesize the phosphorylated form, C16-E4pY, in cancer cells, which induced the selective death of kinase-overexpressing cancer cells. C16-E4Y also exhibited antitumor activity in vivo. In the last decade, the in situ synthesis of a therapeutic agent in living cells has become a potential and powerful approach for therapeutic applications.[37−39] The present study took advantage of an overexpressed enzyme and ATP in cancer cells to achieve the in situ synthesis of a self-assembling peptide amphiphile in cancer cells without the addition of a catalyst. The in situ synthesis of a drug and molecular self-assembly in cancer cells affords a new potential therapy for treating cancer.

EXPERIMENTAL SECTION

Preparation of Peptide Amphiphiles

Peptide amphiphiles used in the study were prepared via a solid-phase synthesis. They were purified by HPLC and confirmed by MALDI-TOF/MS.

Gelation Test

Peptide amphiphiles were dissolved in PBS(+) (containing Ca^{2+} (0.90 mM) and Mg^{2+} (0.49 mM)) at 1.0 wt % with heating and then slowly cooled at room temperature. Gel formation was evaluated by tilting the glass vials.

TEM Observation

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 at an acceleration voltage of 200 kV.

Tyrosine Kinase Activity Assay

The tyrosine kinase activity of cells was determined using the Universal Tyrosine Kinase Assay Kit according to the manufacturer's protocol.

Cytotoxicity Assay

Cells were seeded into a 96-well plate. After incubation for 24 h, the medium was exchanged with a medium containing a peptide amphiphile, and the cells were incubated for 20 h. The cell viability was determined using the WST-8 reagent according to the manufacturer's protocol.

Tyrosine Kinase Inhibitor Assay

Afatinib or erlotinib was added 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.

Localization of Peptide Amphiphiles in Cells

A431 cells were seeded on a 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 ER-tracker red. The cells were observed using CLSM.

Apoptosis and Necrosis Detection

Cells were incubated for 24 h on a dish. The cells were incubated with a medium containing C16-E4Y (0.08 wt %) for 1 h. The apoptosis and necrosis were detected by CLSM using the Apoptotic/Necrotic/Healthy Cell Detection Kit according to the manufacturer's protocol.

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). A431-xenografted mice were prepared by subcutaneously injecting a suspension of A431 cells into the flank. PBS containing 1.5 wt % C16-E4Y was directly injected into the tumor three times a week for 3 weeks. The tumor volumes were calculated using the following formula:

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

Mice were sacrificed 4 weeks after inoculation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.2c00301.

Materials; experimental details and supplementary results including MS data and HPLC data for compounds; confirmation for the phosphorylation and the gelation of the peptide amphiphiles; TEM and CLSM observation of self-assembled fibers of the peptide amphiphiles; cytotoxicity of compounds; CLSM observations; and FRAP assay and apoptosis/necrosis assay (PDF)

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Author Contributions

CRediT: **Kenta Morita** investigation, methodology, writing-original draft; **Kanon Nishimura** investigation, methodology; **Shota Yamamoto** investigation, methodology; **Natsumi Shimizu** investigation; **Tomoko Yashiro** investigation; **Ryoko Kawabata** investigation; **Takashi Aoi** methodology, supervision; **Atsuo Tamura** methodology; **Tatsuo Maruyama** conceptualization, supervision, writing-original draft, writing-review & editing.

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Notes

The authors declare no competing financial interest.

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