Bioconjugate Chemistry

Communication

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Development of Cell-Penetrating R7 Fragment-Conjugated Helical Peptides as Inhibitors of Estrogen Receptor-Mediated Transcription

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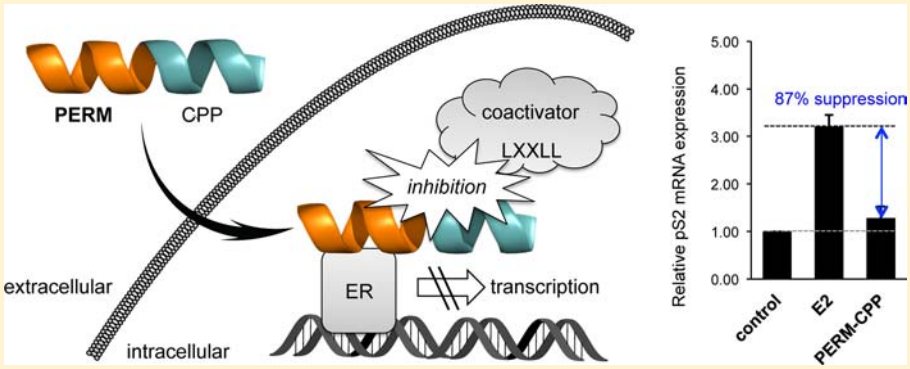
Supporting Information



ABSTRACT: The heptaarginine (R7)-conjugated peptide **5** was designed and synthesized as an inhibitor of ER-coactivator interactions and ER-mediated transcription at the cellular level. The R7-conjugated peptide **5** was able to enter ER-positive T47D cells efficiently, and treatment with 3 μM of **5** downregulated the mRNA expression of pS2 (an ER-mediated gene) by 87%.

INTRODUCTION

Breast cancer is the most common cancer in women, and its incidence is increasing from year to year. The estrogen receptor (ER), which is a ligand-inducible transcription factor and a member of the nuclear receptor superfamily, is often overexpressed in the tissues of breast cancer patients and promotes the estrogen-dependent proliferation of cancer cells.[1−3] Several ERα antagonistic drugs, such as tamoxifen and nonsteroidal selective ER modulators, have been developed as treatments for breast cancer.[4−8] Among those antagonists, tamoxifen acts via the competitive inhibition of 17β-estradiol (E2) and is the most widely used drug for treating breast cancer.[9,10] However, tamoxifen has agonistic effects on ERα in uterine cancer cells and increases the risk of endometrial cancer.[11,12] In addition, it activates the protein kinase B (Akt) signaling pathway by binding to a particular ER variant, resulting in the inhibition of apoptosis in cancer cells.[13,14] Therefore, novel drug candidates with different mechanisms of action have long been desired. ER-mediated gene activation is induced by the binding of E2 to the ER ligand-binding domain and the subsequent binding of the consensus LXXLL helical motif[15] (L: leucine, X: any amino acid residue) of the coactivator with the ER surface.[16,17] Helical peptides containing the above-mentioned consensus sequence have been demonstrated to inhibit ER-coactivator interactions, and they are also considered to be drug candidates for reducing ER-mediated transactivation. Various helical peptides have been reported as inhibitors of ER-coactivator interactions.[18−22] The peptidomimetic estrogen receptor modulators (PERMs), specifically, PERM-1 and PERM-3 [with two mutation: Lys(1) → Arg(1) and Leu(7) → Npg(7)] reported by Burris et al. exhibited particularly potent inhibitory activity against ER-coactivator interactions.[19,20] However, only a few peptide-based ER-transcription inhibitors that exhibit potent activity at the cellular level have been reported[23,24] because of the low cell permeability of such peptides. Thus, we assumed that the conjugation of PERM with cell-penetrating peptides such as oligoarginines and their derivatives[25,26] might solve the problems surrounding the development of novel peptide-based transcriptional inhibitors (Figure 1). In this communication, we describe the synthesis of heptaarginine (R7)-conjugated PERM as inhibitors of ER-signaling at the cellular level (Table 1). Specifically, we synthesized R7-conjugated helical peptides and evaluated their cellular uptake, ability to inhibit transcription in ER-positive T47D cells, ability to inhibit ER-coactivator interactions, and their preferred secondary structures (by assessing their CD spectra).

RESULTS AND DISCUSSION

Synthesis of Peptides. The N-terminal-free peptides **1**−**7** and their N-terminal fluorescein (FAM) labeled peptides were

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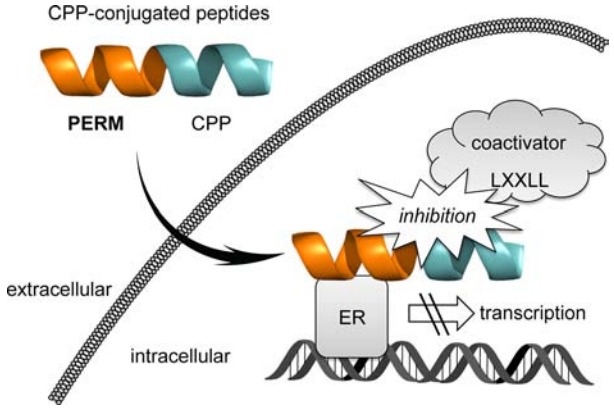


Figure 1. Illustration of the mechanism by which the CPP-conjugated peptides inhibited ER-coactivator interactions at the cellular level.

synthesized using microwave-assisted Fmoc-based solid phase methods, respectively. All of the peptides were purified by reversed-phase high performance liquid chromatography and were characterized using electrospray ionization time-of-flight mass spectrometry (Supporting Information).

Biological Evaluations. First of all, we evaluated the cellular uptake of fluorescein-labeled peptides (green; 1 μM) into ER-positive T47D cells (incubated for 3 h) using confocal laser scanning microscopy (CLSM, Supporting Information) as shown in Figure 2. The R7-unconjugated peptide **FAM-2** (an N-terminal fluorescein-labeled version of peptide **2**) was completely unable to enter the ER-positive T47D cells, whereas the R7-conjugated peptide **FAM-5** passed through the cell membrane efficiently and was distributed in the cytoplasm and nucleus. This difference in the cell-penetrating abilities of the molecules was solely due to the presence/absence of R7 conjugation. The R7-conjugated peptide **FAM-4** also exhibited cell permeability (Supporting Information).

Then, we evaluated the ability of the R7-conjugated peptides to inhibit ER-mediated transcription. Transcriptional analysis of an ER target gene (pS2) was carried out using T47D cells that had been incubated with one of the peptides (3 μM) in the absence or presence of 10 nM E2 for 24 h. The mRNA expression of pS2, which is the one gene whose expression is upregulated by E2, was analyzed using the quantitative polymerase chain reaction (Supporting Information). The relative pS2 mRNA expression levels of the cells treated with each peptide are summarized in Figure 3. The R7-unconjugated (nonmembrane-penetrating) peptides **1**−**3** and the heptaarginine (YR7)[27] peptide did not inhibit transcription. On the other hand, the mRNA expression of pS2 was significantly decreased (by 87%) by the addition of 3 μM of the R7-conjugated peptide **5**. Treatment with 3 μM of the R7-conjugated peptide **4** did not suppress the mRNA expression of pS2 at all, but treatment with 10 μM of **4** decreased it by 95% (Supporting Information). Conversely, treatment with the R7-conjugated peptide **6** at concentrations ranging from 3 μM to 10 μM did not induce any significant reduction in ER-mediated transcriptional activity. These results demonstrated that the R7-conjugated peptides **4** and **5** were able to exhibit antagonistic effects on ER-mediated transcription at the cellular level, and **5** displayed particularly potent inhibitory activity.

The inhibitory activity of peptides **4** and **5** against ER−coactivator interactions were evaluated using EnBio receptor cofactor assay systems (RCAS) for ERα (Fujikura Kasei Co., Ltd.) according to the manufacturer's instructions (Figure 4). The R7-unconjugated peptides **1** and **2** demonstrated strong activity against ERα−coactivator interactions. While the activities of the corresponding R7-conjugated peptides **4** and **5** were reduced, peptide **5** still demonstrated strong activity (IC_{50}: 94 nM). These results indicated that peptides **4** and **5** suppress ER-mediated transcription by inhibiting ERα−coactivator interactions. The R7-conjugated peptide **5** exhibited stronger inhibitory activity against ERα−coactivator interactions than **4**, and therefore, **5** was able to suppress ER-mediated transcription more efficiently than **4**, even at the cellular level.

The dominant conformations of peptides **1**−**6** were analyzed by assessing their CD spectra in 20% aqueous 2,2,2-trifluoroethanol (TFE) solution (Figure 5). The CD spectra of peptides **1** and **2**, and those of their R7-conjugated peptides **4** and **5**, displayed negative maxima at around 208 and 222 nm, indicating that all of the peptides formed stable right-handed α-helical structures. These findings suggested that the R7 fragment did not disrupt helix formation. On the other hand, the SRC-1 peptide **3** and its R7-conjugated form **6** were found to be composed of random coil structures rather than α-helices. These results indicated that peptides require stabilized helical structures in order to possess significant inhibitory activity against ER-coactivator interactions.

■ CONCLUSION

In conclusion, we developed heptaarginine (R7)-conjugated PERM as molecules that could be used to suppress ER-mediated transcription at the cellular level. The R7-conjugated peptides were able to enter ER-positive T47D cells efficiently, and one of them, peptide **5**, downregulated the mRNA expression of pS2 by 87% at a dose of 3 μM. Furthermore, **5** displayed strong inhibitory activity (IC_{50}: 94 nM) against ER−coactivator interactions. Although the inhibitory activity of the R7-conjugated peptide **5** against ER−coactivator interactions was slightly decreased compared with that of the R7-unconjugated peptide **2** (IC_{50}: 13 nM), **5** still exhibited potent activity. The dominant conformations of the peptides were analyzed based on

Table 1. Sequences of Peptides 1−7

peptide	sequence
PERM-1 (**1**)	H-Lys-*cyclo*(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-NH_2
PERM-3 (**2**)	H-Arg-*cyclo*(D-Cys-Ile-Leu-Cys)-Arg-Npg[a]-Leu-Gln-NH_2
SRC-1[b] (**3**)	H-His-Lys-Ile-Leu-His-Arg-Leu-Leu-Gln-NH_2
PERM-1-R7 (**4**)	H-Lys-*cyclo*(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$(Gly)_3$-$(Arg)_7$-NH_2
PERM-3-R7 (**5**)	H-Arg-*cyclo*(D-Cys-Ile-Leu-Cys)-Arg-Npg-Leu-Gln-$(Gly)_3$-$(Arg)_7$-NH_2
SRC-1-R7 (**6**)	H-His-Lys-Ile-Leu-His-Arg-Leu-Leu-Gln-$(Gly)_3$-$(Arg)_7$-NH_2
YR7 (**7**)	H-Tyr-$(Arg)_7$-NH_2

[a]Npg: neopentylglycine. [b]The LXXLL motif of the coactivator.

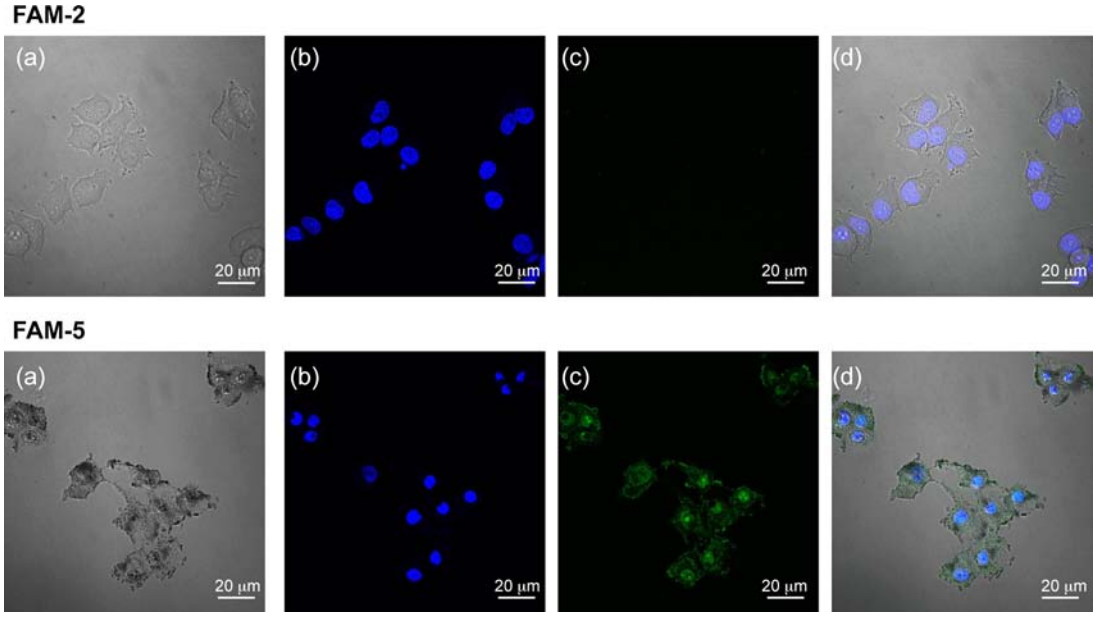


Figure 2. CLSM images of T47D cells that had been treated with **FAM-2** or **FAM-5** (peptide concentration: 1 μM, incubation time: 3 h). (a) Bright-field images, (b) nuclei stained with Hoechst 33342 (blue), (c) the intracellular distribution of the FAM-conjugated peptides (green), and (d) merged images.

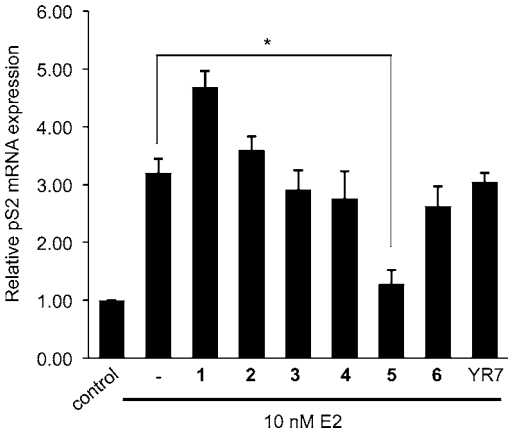
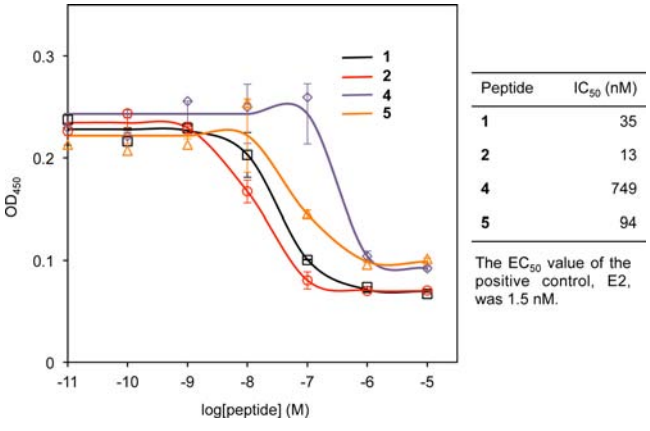


Figure 3. Inhibition of ERα-mediated gene expression in T47D cells. Peptide concentration: 3 μM. The error bars represent standard deviation, $n = 3$. *$p < 0.05$.



Peptide	IC_{50} (nM)
1	35
2	13
4	749
5	94

The EC_{50} value of the positive control, E2, was 1.5 nM.

Figure 4. IC_{50} values of peptides against ERα–cofactor interactions according to RCAS.

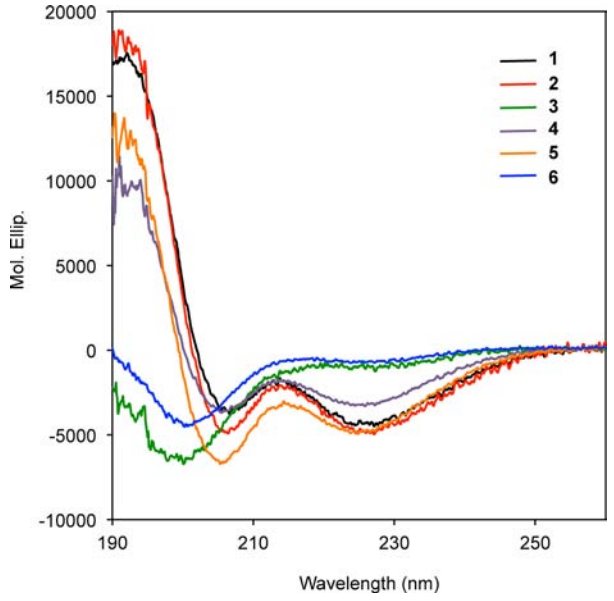


Figure 5. CD spectra of peptides **1**–**6** in the 190–260 nm region. Peptide concentration: 100 μM in 20% aqueous TFE solution.

their CD spectra, and it was found that **5** formed a right-handed α-helical structure similar to that of the R7-unconjugated peptide **2**. The R7-conjugation of the PERM did not disrupt their helical structures. These results indicate that the conjugation of PERM to R7 would aid the development of novel inhibitors of ER-mediated transcription at the cellular level. The derivatization of further helical peptides and detailed studies of their inhibitory mechanisms are currently underway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Information about the synthesis and purification of the peptides and the protocols of the in vitro assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Akt, protein kinase B; CD, circular dichroism; CLSM, confocal laser scanning microscopy; ER, estrogen receptor; E2, 17β-estradiol; PERM, peptidomimetic estrogen receptor modulators; RCAS, receptor cofactor assay systems; R7, heptaarginine; TFE, 2,2,2-trifluoroethanol

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