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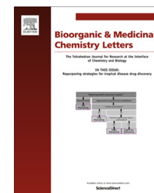

Development of a peptide-based inducer of nuclear receptors degradation

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ABSTRACT

A peptide-based protein knockdown system for inducing nuclear receptors degradation via the ubiquitin–proteasome system was developed. Specifically, the designed molecules were composed of two biologically active scaffolds: a peptide that binds to the estrogen receptor α (ERα) surface and an MV1 molecule that binds to cellular inhibitors of apoptosis proteins (IAP: cIAP1/cIAP2/XIAP) to induce ubiquitylation of the ERα. The hybrid peptides induced IAP-mediated ubiquitylation followed by proteasomal degradation of the ERα. Those peptides were also applicable for inducing androgen receptor (AR) degradation.

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The estrogen receptor (ER) is a member of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors. There are two different forms of the ER, ERα and ERβ,[1,2] and ERα is often overexpressed in the tissues of breast cancer patients and promotes the estrogen-dependent proliferation of cancer cells.[3,4] ER transcription is activated by the binding of 17β-estradiol (E2) to the ER ligand-binding domain and the subsequent binding of a coactivator and the consensus LXXLL helical motif (L: leucine, X: any amino acid residue) to the ER surface.[5–7] A variety of antagonists such as tamoxifen and raloxifene, which are competitive inhibitors of E2, are widely used to treat breast cancer.[8,9] However, some of these antagonists have agonistic effects on ERα in uterine cancer cells and increase the risk of endometrial cancer.[10,11] Therefore, new inhibitors with different mechanisms of action are required as novel drug candidates. To this end, we have recently identified two types of ERα inhibitors. We found that the Peptidomimetic Estrogen Receptor Modulator (PERM) derivative PERM3-R7 inhibits ER-coactivator interactions (Fig. 1a).[12,13] PERM3 is an LXXLL-like mimetic of the Steroid Receptor Coactivator 1 (SRC-1) that interacts with the ERα surface,[14,15] and R7 is a hepta-arginine fragment that enhances the cellular permeability of PERM3. PERM3-R7 could form a stable α-helical structure and potently inhibit ER-mediated transcription and downregulates the mRNA expression of pS2 (an ER-mediated gene whose expression is upregulated by E2) at the cellular level.[12,13] The other molecule we identified is able to inhibit ERα activity via the small molecule-based ERα degradation method,[16–18] which is also known as the protein knockdown strategy (Fig. 1b).[19–21] That is to say, a hybrid molecule composed of a tamoxifen derivative (4-hydroxytamoxifen; 4-OHT) that binds to the ERα, and bestatin (BS), which binds to cellular inhibitor of apoptosis protein 1 (cIAP1), is able to lead to ubiquitylation and subsequent proteasomal degradation of the ERα via the ubiquitin–proteasome system (UPS).

Therefore, we speculated that a molecule in which the peptide PERM3-R7 was conjugated to one of the small molecules that bind to IAP (e.g., cIAP1, cIAP2, or XIAP) would also be able to induce UPS-mediated ERα degradation. To date, various peptides have been reported to inhibit protein-protein interactions,[22–24] and these proteins could be used in a peptide-based protein knockdown system.[25] It would be easy to identify inducers of protein degradation because most peptides interact with the surfaces of their target proteins. Therefore, this peptide-based protein knockdown strategy might also be applicable to some types of proteins such as receptor proteins the ligand of which is not determined and proteins the ligand of which is buried. In this communication, we describe the synthesis of peptide-based ERα degradation inducers by connecting peptides that bind to the ERα surface with the ligand MV1,[26,27] which binds to IAP, to induce protein ubiquitylation (Fig. 2). We also applied these hybrid peptides for an androgen

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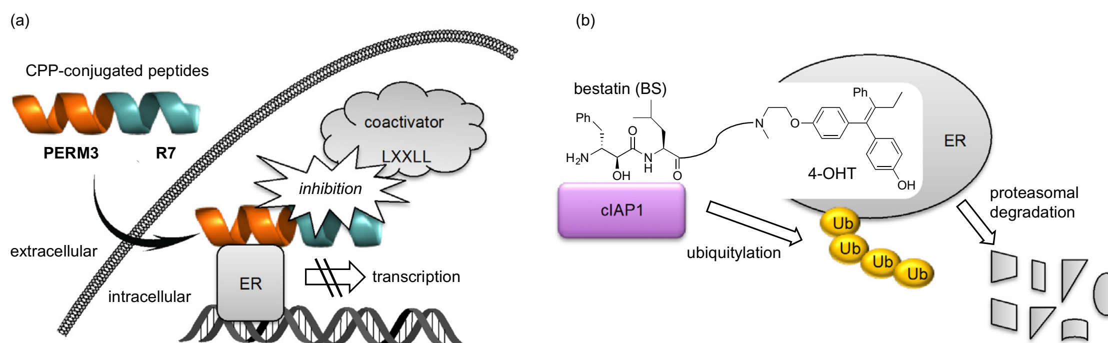

Figure 1. (a) The inhibition of ER-coactivator interactions at the cellular level using a cell-penetrating peptide (CPP)-conjugated peptide (b) ER degradation strategy using a ligated hybrid molecule.

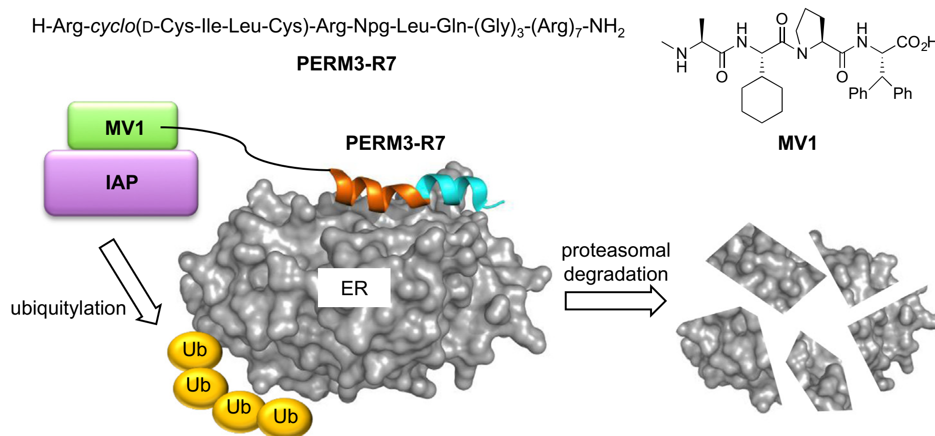

Figure 2. Illustration of the mechanism by which the peptide-based ER degradation system examined in this study works.

receptor (AR) degradation, because the transcriptional activity of AR is also mediated through its interaction with SRC-1, and AR inhibitors are drug candidates for treating prostate cancer.

First, we designed three types of peptides: peptide **1**, in which MV1 was attached to the N-terminal end of PERM3-R7 via an ethylene glycol-based linker; peptides **2**–**4**, in which the Arg(1) residue in the PERM3-R7 sequence was substituted for Lys(1) and MV1 was conjugated to the side-chain ε-position of the Lys(1) residue of PERM3-R7 via a ethylene glycol-based linker ($n = 0$, 1, or 2); and peptide **5**, in which the linker of peptide **4** was substituted for five β-alanine residues (Fig. 3). Peptides **1**–**5** were synthesized using Fmoc-based solid-phase methods. The following describes a representative coupling and deprotection cycle. First, NovaPEG Rink amide resin was soaked for 1 h in CH_2Cl_2. After the resin had been washed with *N*-methyl-2-pyrrolidone (NMP), Fmoc-amino acid (4 equiv), and HBTU (4 equiv) dissolved in NMP were added to the resin. Then, DIPEA (4 equiv) and 0.1 M HOBt in NMP were added for the coupling reaction. Deprotection was carried out using 20% piperidine in DMF. After the peptide synthesis, the resin was suspended in cleavage cocktail (94% TFA, 2.5% water, 2.5% 1,2-ethanedithiol, 1% triisopropylsilane) for 4 h at rt. The TFA solution was evaporated to a small volume under a stream of N_2 and dripped into cold ether to precipitate the peptides. The peptides were dissolved in DMSO and aqueous NH_3 (enough to make the solution basic) for a disulfide bond formation. All of the peptides were purified by reversed-phase high-performance liquid chromatography.[28]

The ERα-protein degradation activity (in MCF-7 breast cancer cells) of the synthesized peptides was evaluated by Western blot analysis. Peptide **1**, in which MV1 was conjugated to the N-terminal backbone of PERM3-R7, did not reduce the level of ERα protein, and cytotoxic effects were observed after treatment with 10 μM peptide **1** (Fig. 4a).[29] Peptides **2** and **3**, in which MV1 was conjugated to the ε-position of the Lys(1) residue of PERM3-R7 via an ethylene glycol-based linker ($n = 0$ for **2**, $n = 1$ for **3**), also did not degrade the ERα protein (Fig. 4b and c). The level of cIAP1 protein was reduced by treatment with 1.0 μM peptide **3**, indicating that **3** activates the auto-ubiquitylation and subsequent proteasomal degradation of cIAP1 as observed in the presence of the 4-OHT-based degradation inducer (Fig. 4c).[16–18]

On the other hand, peptide **4**, which contained an elongated linker ($n = 2$), dose-dependently reduced the levels of ERα protein and cIAP1 (Fig. 5a). However, >6.0-μM doses of **4** had cytotoxic effects. Intriguingly, peptide **5**, in which the linker of **4** was substituted for β-alanine residues, also acted as an inducer of ERα-degradation (Fig. 5b). Although **5** exhibited lower ERα-degradation activity than **4**, peptide **5** did not have any cytotoxic effects, even at a dose of 20 μM. Furthermore, the reductions in the levels of ERα and cIAP1 induced by **5** were suppressed by MG132, a proteasome inhibitor, indicating that **5** induced the proteasomal degradation of ERα and cIAP1. We also evaluated the AR-degradation activity of peptides **4** and **5**, and as expected, the concentration of AR protein was reduced by treatment with either peptide (Fig. 5a and b). The reductions in the AR concentration induced by these peptides were also abrogated by MG132, suggesting that AR is also degraded by these proteasomes. On the other hand, the level of the aryl hydrocarbon receptor (AhR), which is a ligand-inducible transcription factor that is regulated by the coactivator CBP/p300,[30] was not

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